Volatile Organic Compound and Microbiome profiling in patients with colorectal cancer, their spouses and first degree relatives

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Dedication

This thesis is dedicated to my late uncle, Chris Sparey. He lost his fight with colorectal cancer, but managed to lose with dignity and a smile.

Declarations

I, Michael McFarlane, declare that this thesis has not been submitted for a degree at any other University.

The work presented here, including data processing and data analysis, was carried out by the author, except in the cases outlined below:

- Data processing of raw LC-MS-FAIMS data by Aditya Malkar at Owlstone (chapters 5 and 6)

- VOC statistical analysis performed in collaboration with Matthew Thomas and Richard Savage (chapters 4, 5 and 6)

- 16s bioinformatics analysis performed in collaboration Andrew Millard (chapters 7 and 8)

Synopsis

Colorectal cancer (CRC) is the one of the commonest causes of cancer and cancer related death worldwide. Its aetiology is linked to a number of reversible and irreversible genetic and environmental factors, including age, sex, genetics, smoking and diet. There has been a drive in recent years for non-invasive biomarkers for many malignant and non-malignant diseases across multiple medical specialties. One of the areas of interest is the detection of volatile organic compounds (VOCs) in various bodily substances by means such as mass spectrometry and electronic noses. CRC patients have been shown to be distinguishable from healthy controls using urinary VOC detection in several studies, including two published by the research group at UHCW and the University of Warwick. There has also been much interest in recent years into the role that the intestinal microbiome plays in health and disease in humans.

The aim of this thesis was to characterise the urinary VOC and stool microbiome profiles of CRC patients, their spouses and first degree relatives. The aim being to determine whether the urinary VOC profiles could be distinguished using this technology and to try and better understand the underlying mechanism which lead to CRC carcinogenesis. The first degree relatives and spouses were selected as “common gene pool” and “shared environment” control groups respectively.

This work was done using an LC-FAIMS-MS hybrid machine to detect urinary VOCs and 16s RNA sequencing using an Illumina Miseq platform. Comparisons were also made between pre-treatment and post-treatment CRC samples to try and determine if there was any change in either VOC or microbiome profiles after CRC treatment.

The urinary VOC profiles of CRC subjects could be distinguished from both sets of healthy controls using a 5-fold cross validation and sparse logistics regression and Random Forrest statistical classifiers, achieving sensitivities of 63-69%, specificities of 64-69% and AUC 0.71-0.72. No statistically significant differences could be found in the urinary VOC profiles of pre-operative and post operative samples.

Microbiome analysis revealed over 1300 operational taxonomic units (OTUs), with a similarity of >93% between the CRC samples and the control groups, with significantly different bacterial abundances identified in only 82 OTUs (6.2%), mainly Clostridiales bacteria. Pre-treatment and post-treatment sample analysis revealed differences of 17 (3%) and 22 (4%) OTUs at 3 and 6 months respectively, again principally clostridiales.

This thesis provides further data on the microbiome composition in CRC. It also provides further proof of the utility of urinary VOCs, for the first time here using LC-FAIMS-MS technology, a variant of the previously utilised FAIMS technology, as a non-invasive biomarker for CRC.

Abbreviations

ANOVA: Analysis of Variance

APC: adenomatous polyposis coli

AUC: area under the curve

BCSP: Bowel cancer Screening Programme

BMI: body mass index

Bp: Base pairs

BSS: Bowel Scope Screening

CEA: Carcino Embryonic Antigen

COPD: Chronic Obstructive Pulmonary Disease

COX2: Cyclo-Oxgenase-2

CRC: Colorectal cancer

CT: Computerised Tomography

DNA: deoxyribonucleic acid

DCIS: ductal carcinoma in situ

E-nose: Electronic nose

FAIMS: Field Asymmetric Ion Mobility Spectrometry

FAP: Familial Adenomatous Polyposis

FIT: faecal Immunohistochemical Test

FOBT: faecal occult blood test

FT-ICR-MS: fourier transform-ion cyclotron resonance mass spectrometry

GC-MS: Gas Chromatography and Mass Spectrometry

GI: Gastrointestinal

GP: General Practitioner

GWAS: Genome wide association studies

HCC: Hepatocellular carcinoma

HLA: Human Leucocyte Antigen

HNPCC: Hereditary Non-Polyposis Colorectal Cancer

HNSCC: head and neck squamous cell cancer

HRT: Hormone Replacement therapy

HSD: honest significance difference

IBD: inflammatory bowel disease

IMS: Ion Mobility Spectrometry

LC: Liquid Chromatography

MDT: Multi-Disciplinary Team

MRI: Magnetic Resonance Imaging

MS: Mass Spectrometry

MUTYH: mutY homolog

OTU: Operational Taxonomic Unit

PCR: polymerase chain reaction

PSA: prostate specific antigen

PTR-MS: Proton Transfer Reaction Mass Spectrometry

ROC: Receiver Operator Curve

ROS: Reactive Oxygen Species

RNA: RiboNucleic Acid

SIFT-MS: Selected Ion Flow Tube Mass Spectrometry

SPME-GC: solid phase microextraction-gas chromatography

SD: Standard Deviation

TAE: Tris-acetate

TIC: total ion current

TNM: Tumour Node Metastases

UK: United Kingdom

UHCW: University Hospitals Coventry and Warwickshire

USS: ultrasound scan

VOCs: Volatile Organic Compounds

2WW: 2 week wait

CHAPTER 1

Introduction

1.0 Introduction

Cancer is a leading cause of death worldwide irrespective of the socioeconomic status of individual countries. The number of cancer cases is expected to grow rapidly in line with population increases, and the greater prevalence of lifestyle risk factors, such as tobacco use, physical inactivity and obesity, particularly in low and middle income countries. Colorectal cancer (CRC) is one of the most common types of cancer, and cause of cancer-related death worldwide, with 1 in 20 people developing CRC in their life time.

Currently diagnosis of CRC, in the United Kingdom, is made via 3 main routes; Emergency presentation with a complication of the primary tumour, such as bowel obstruction, via the two week wait pathway, where patients with symptoms suggestive of CRC are referred urgently to hospital for investigation, and via the Bowel Cancer Screening Programme, where asymptomatic individuals undergo a screening test based on Faecal occult blood testing. As will be discussed later in this thesis, the accuracy of the FOB test is poor, and the test itself has a relatively poor uptake due to the nature of the sample involved. There has been a recent quest for a more reliable and acceptable method of screening for CRC. This has included analysis of the profile of volatile organic compounds (VOCs) in various bodily secretions of CRC patients using numerous different technologies. VOCs are normal by products of an individuals metabolism, which are believed to be perturbed in disease states such as CRC. The analysis of CRC derived VOCs is part of a much wider field of study in the area of VOCs in health and disease, including

malignant and non-malignant conditions, across many body systems, including respiratory, gastrointestinal, metabolic and renal.

This thesis will be a continuation of research I have previously carried out on the urinary VOC profiles of CRC patients. This study discussed later in this thesis found that CRC patients were distinguishable from healthy controls with a sensitivity of 83% and specificity of 60%. This thesis, though independent of this previous work, follows on from it. The primary aim is to analyse the urinary VOC patterns, and the faecal microbiomes, of patients with CRC, their first degree relatives and an individual who lives in the same environment as them. Urinary VOC screening could represent a more acceptable screening medium to patients than the current faecal based tools in the screening for CRC.

The aim of this analysis is to determine whether urinary VOC profiles of CRC patients are unique, compared to environmental (co-habitor), and genetic controls (first degree relative). The previous studies had used healthy controls completely distinct from the CRC subjects.

The urinary profiles of VOCs before, and after, tumour resection will also be studied to determine whether any changes in the VOC profile occurs after removal of the primary lesion.

The composition of the microbiome within all 4 groups, the initial CRC patient specimens, the post-treatment CRC patient specimens, the co-habitors and the relatives, will also be studied using 16s RiboNucleic Acid (RNA) analysis of stool

samples. This is with the aim of elucidating any differences in the intestinal microbiome between the groups. Additionally it should provide further understanding of the changes which occur in the microbiome after CRC surgery.

The combination of these experiments will hopefully allow greater understanding of the potential role of urinary VOCs as a non-invasive biomarker in the screening for CRC and the variation of the faecal microbiome across the main study groups.

As this thesis will cover the aetiology of CRC, the VOC profiling of malignancy, including CRC, and the microbiome of patients with CRC, each of these areas will be discussed individually in the following chapter.

CHAPTER 2

Colorectal Cancer, Volatile Organic Compounds and the Microbiome

2.1. Colorectal Cancer

2.1.1. Epidemiology

2.1.1.1. Incidence

Globally, CRC is the third and second commonest cancer in males and females respectively, with incidence and mortality appearing to be on the increase. In 2008, an estimated 1.2 million people were diagnosed with CRC (1). By 2012 this number had increased to 1.4 million (2). Worldwide, the age standardised rate for CRC incidence is 17.3 per 100, 000 and cumulative CRC risk from birth to age 74 years is 0.9%. CRC incidence is higher in men, with an age standardised male to female ratio of 1.4: 1 (1). Within the United Kingdom (UK), CRC is the fourth most common cancer overall, and the third most common in both men and women, with 41,100 cases diagnosed in 2013 (3).

Despite its global presence, there is marked variation in CRC incidence rates worldwide. Developed or higher income countries account for almost two-thirds of CRC cases, but the incidence rates in these countries are, for the most part stable or declining. The incidence rates in developing countries and those with historically low rates, such as those in Latin America, Asia and Eastern Europe are, however, rising (1, 2). These increases are believed to be due to the effects of adopting a more westernised diet, reduced physical activity and increased tobacco use (4).

The decreasing incidence rates in higher income countries have been attributed to screening programmes for CRC, which result in the removal of pre-cancerous lesions and polyps, and also a reduction in risk factors, particularly smoking. Despite these overall falling incidence rates, higher income countries have been experiencing an increase in the incidence rates for those aged less than 50 years, for whom screening is not currently recommended. The underlying reasons for this rise have not been established (4).

Within the UK, bowel cancer incidence rates are increasing, with a 14% increase since the 1970s. This is likely to be due, in part, to the implementation of the bowel cancer screening programme and an increase in lesion detection (5).

2.1.1.2. Mortality

Globally, mortality figures from CRC continue to increase in line with the rising incidence. In 2008, there were 668,000 CRC related deaths, and in 2012 there were 693,900 (1, 2). Overall, it is the fourth leading cause of cancer deaths, accounting for 8% of all cancer deaths worldwide. In the UK, it is the second most common cause of cancer death after lung cancer, with 15,900 people dying in 2014 (6).

In more developed countries, such as the US and many European countries, despite a higher incidence rate, mortality rates are decreasing. Again, this is attributed to screening programmes detecting the disease at earlier, more treatable stages, and also to the vast improvement made in treatments (7).

Conversely, countries with rising incidence and mortality rates e.g. Brazil, Chile, Romania and Russia, have increasingly westernised diets and life style risk factors, but have relatively less resources for screening and treatment, resulting in poorer mortality statistics (4).

The UKs mortality from CRC has been falling since the 1970s, with 16,200 dying in 2012. Over the last decade, the mortality has reduced by 14%. The UK currently ranks the 10th lowest for males and 14th lowest for females in terms of CRC mortality (6).

The mortality rate of CRC is approximately half of the incidence rate, giving it a relatively good prognosis. The 5 year prevalence of CRC worldwide is approximately 3.26 million (1) whilst in the UK it is 91,777. The UK 5 year survival is 59%, and 57% at 10 years (8).

2.1.2. Aetiology

Epidemiological studies have identified numerous factors which increase the risk of CRC, but also many which decrease CRC risk. Those such as personal or family history of CRC, along with history of inflammatory bowel disease cannot be modified, but lifestyle risk factors such as smoking, alcohol use, diet and physical inactivity, can be.

2.1.2.1. Age

Age is a major non-modifiable risk factor for both CRC and CRC mortality. In the UK, 43% of bowel cancer was diagnosed in those aged >75 years, whilst 95% were diagnosed in those aged 50 and over. The bowel cancer screening programme (BCSP) was introduced in England in 2006, and has subsequently been rolled out across the whole of the UK. This led to a 14% increase in CRC incidence for those aged 60-69 years between 2004-2006, and 2008-2010 (6).

2.1.2.2. Sex

Worldwide, and in the UK, men are at greater risk of CRC than women, but the reasons for this are not well understood. They have been postulated to be related to hormonal, genetic and molecular interactions, as well as environmental risk factors (9).

2.1.2.3. Genetic predisposition

Approximately 5% of CRC can be directly attributed to genetic predisposition. The main inherited genetic syndromes predisposing to CRC are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch Syndrome.

FAP accounts for approximately 1% of CRC. It shows an autosomal dominant inheritance pattern and is due to a germline mutation in the adenomatous polyposis coli (APC) gene, located on chromosome 5q21. FAP is characterised by

the development of hundreds to thousands of gastrointestinal polyps, which undergo near inevitable progression to CRC by the age of 40-45 years (10). The treatment of these patients involves genetic screening of families, endoscopic surveillance and colectomy.

HNPCC meanwhile accounts for approximately 1-4% of CRC, which also shows an autosomal dominant pattern of inheritance and is due to mutations in DNA mismatch repair genes including MSH2, MLH1, MSH6 and PMS2. These mutations lead to microsatellite instability and subsequent carcinogenesis. Additionally, HNPCC confers a higher risk of other gastrointestinal (GI) cancers, such as gastric, but also endometrial cancer. The lifetime risk for developing CRC with HNPCC is approximately 80% (11).

Other syndromes associated with increased CRC risk are Peutz-Jegher's syndrome and mutY homolog (MUTYH) associated polyposis. Peutz-Jegher's syndrome is a rare autosomal dominant condition caused by mutations in the STK11 gene. Patients develop characteristic hyperpigmentation of the fingers, toes and lips and are at increased risk of developing hamartomatous polyps of the GI tract. MUTYH-associated polyposis is an autosomal recessive condition caused by mutation in the base excision repair gene MUTYH, resulting in multiple colorectal adenomas which can progress to CRC (7).

2.1.2.4. Family History

Non-genetic susceptibility CRC, or sporadic CRC, does still appear to have a genetic component to it. Family history is an important risk factor for the development of CRC, even without the presence of genetic predisposition. A first degree relative, such as a child, parent or sibling, having suffered from CRC leads to a doubling of the risk of developing CRC by the age of 70 (equating to a 1 in 30 risk). This risk is further increased if multiple relatives are affected, or diagnosis occurs at younger than 60 years of age. This increase has been suggested to be due to an interaction of genetic and environmental causes (12).

It has been found that adopted children are less likely to develop CRC if their biological parents did not have CRC, compared to if they did. Also children with adoptive parents who suffer from CRC are not at increased risk of developing CRC (13). This would suggest that the genetic factors, rather than environmental factors, underpin the increased family history risk of CRC.

Genome wide association studies (GWAS) have been conducted to examine for markers of CRC risk. These studies initially showed only a few low penetrance markers, and failed to identify specific genes. However, further, more powerful studies have identified multiple low penetrance susceptibility loci which would account for a higher proportion of CRC. This would support the theory that sporadic familial CRC results from the cumulative effects of multiple low penetrance genes. These familial clusters of sporadic CRC may represent

approximately 20% of all CRC, with the remaining 75% of CRC representing true sporadic CRC caused by the accumulation of multiple somatic mutations (14-17).

2.1.2.5. Personal Medical History

A past medical history including any of; adenomatous polyps, previous CRC, inflammatory bowel disease (IBD), gallstones, type 2 Diabetes Mellitus and the metabolic syndrome places people at an increased risk of developing CRC.

- A pooled analysis of adenomas and polyps has shown that 1% of people with adenomas measuring greater than 20millimetres, or adenomas of any size with high grade dysplasia will develop CRC within 4 years of adenoma removal (18). Even the presence of low risk adenomatous polyps increases your risk of developing CRC by 80% compared with people who have no polyps (19).
- IBD (Ulcerative Colitis or Crohn's Disease) conveys an increased risk of developing CRC. This risk is not now thought to be as high as previously believed, but is still 70% higher than the general population (standardised incidence ratio 1.7). Greater risk is found in those with more extensive disease, longer disease duration and younger age at diagnosis. The cumulative risk of CRC were found to be 1%, 2% and 5% after 10, 20 and >20 years of disease duration respectively (20).
- The presence of gallstones confers a 33% increased risk of developing rectal cancers and more than doubles the risk of developing a colonic

adenoma compared to those who do not have gallstones. Interestingly, cholecystectomy does not affect the risk of developing adenoma or CRC (21, 22).

- Several studies have found an increased CRC risk in patients with type II diabetes mellitus, compared to non-diabetics. This risk appears to be 22-30% greater (23-27). CRC risk is also higher by 33-41% in patients with the metabolic syndrome (characterised by a combination of diabetes, hypertension and central adiposity) (28).

2.1.2.6. Obesity

Obesity is a recognised risk factor for developing many forms of cancer, including CRC. Obesity, measured by body mass index (BMI) >30, conveys a 33% increased risk compared to those with normal BMI. This association is stronger for colon cancer than for rectal cancer, and for men compared to women. Waist circumference is also a risk factor for CRC, with larger waist circumference leading to a 46% increased risk in CRC (29).

2.1.2.7. Physical activity

Physical activity has been shown to be strongly protective against the development of CRC, with a 17-24% reduction in risk in the most physically active people compared to the least physically active people (30, 31).

2.1.2.8. Diet

Many dietary components have been implicated in both increased and decreased risk of developing CRC.

2.1.2.8.i. Fruits and vegetables

Non-starchy vegetables and fruit show an inconclusive relationship with CRC in terms of risk. Some studies have shown a reduction in risk with increased consumption (32-34), whereas other studies have shown only a weak, or absent, protective effect (35, 36).

2.1.2.8.ii. Red Meat and Processed Meat

Red meat and processed meat consumption have been linked with an estimated 21% of UK CRC, with an increase in risk of 17-30% per 100-120g/day of red meat intake and 9-50% per 25-50g/day of processed meat intake (37-40). Haem iron, which is found in red meat, is associated with an increased risk of 12% per 1mg/day intake (41). Although, in another cohort study, CRC risk was not found to be linked to whole dietary iron intake (42).

2.1.2.8.iii. Fibre

Dietary fibre is believed to be protective against CRC. 12% of CRC in the UK is linked to consuming less than 23g/day of fibre (43). There is believed to be a 10% reduction in risk per 10g/day of total dietary fibre and cereal fibre, but interestingly there seems to be no association with fruit and vegetable fibre.

Whole grains appear to reduced CRC risk by 20% for every 90g consumed per day (44).

2.1.2.8.iv. Calcium and dairy

Milk and calcium are protective against the development of CRC. Milk reduces the risk of CRC by 9-15% per 200-250g consumed per day (45). Calcium meanwhile, reduces the risk by 8% per 300mg consumed per day, and the risk was found to be 22% lower in the highest calcium intake group, compared to the lowest (46). Calcium supplementation of the diet shows a mixed picture of evidence, with some support for 9% lower risk per 300mg consumed per day, whilst other studies have shown no risk reduction (47, 48).

2.1.2.8.v. Micronutrients

There has been much study into the role of micronutrients into the risk of CRC. Retinol blood levels have been linked with a 37% reduction in CRC for the highest versus the lowest serum levels (49, 50). Vitamin D has been linked with a 15-26% reduction in CRC risk per 10-20ng/ml rise in serum levels (49-52), although, there is also some evidence that it has no effect on CRC risk (42). Beta-carotene (31%), Vitamin E (35%), Vitamin C (40%) and Zinc (20%) have all been linked with reduction in CRC risk for those with the highest intake versus those with the lowest intake (53, 54).

2.1.2.9. Smoking

Cigarette smoking is one of the most common preventable risk factors for the development of many types of cancer, including CRC. It is estimated that 8% of CRC cases are linked to tobacco smoking, with CRC risk greater by 17-21%, compared to non-smokers. The association is stronger for colon cancer than rectal cancer, and for males compared to females. The risk is higher in heavier smokers, with an increase of 7-11% for every 10 cigarettes smoked per day (55, 56).

2.1.2.10. Alcohol

In addition to cigarette smoking, alcohol consumption is another of the commonest preventable risk factors for the development of CRC. An estimated 11% of CRC cases in the UK are linked to alcohol consumption, with an increase in risk of 21% for those consuming 1.5-6 units per day; and 52% for those consuming 6 units or more per day. Overall this equates to an increase of 7% per unit of alcohol consumed per day (57).

2.1.2.11. Medication

Several medications have been found to have a chemoprotective effect against developing CRC. The strongest evidence is for Aspirin and Cyclo-Oxgenase-2 (COX-2) inhibitors, such as celecoxib and rofecoxib (58), although the latter two are no longer in widespread clinical use. Daily aspirin use for 5 years or more conveys a 32-49% risk reduction in developing CRC, compared to those who do not use aspirin (59). It also reduces the risk of developing adenomatous polyps by 17%

(60). Unfortunately, the risks of regular aspirin use, such as GI haemorrhage, mean that at the moment aspirin is not recommended for primary prophylaxis of CRC (61).

Hormone Replacement therapy (HRT) and oral contraceptives have both been shown to have a protective effect against CRC. HRT carries a 16% lower risk for people who have used it, compared to those who have never used it. There is some evidence that current users have better protection than previous HRT users (62-65). Oral contraceptives, meanwhile, convey a 14-19% reduced risk for those who have used them, compared to those that have never used them (60, 66).

There is contradictory evidence for the role of folate. Some studies have found a protective effect against CRC (67), some found no effect (68, 69) and one study found that folate may increase adenoma risk (68).

2.1.3. Diagnosis

Clinical features of CRC depend on the location of the tumour. Approximately 50% of tumours arise in the rectum and sigmoid colon, approximately 30% in the caecum and ascending colon, and the remaining 20% arise between the hepatic flexure and the sigmoid-descending colon junction (70, 71). Left sided and rectal lesions tend to present earlier, due to the narrower lumen of the left sided colon and the more formed nature of the stools within it. The presenting symptoms are usually altered bowel habit, lower abdominal pain, rectal bleeding, and tenesmus (feeling of incomplete evacuation), or sometimes with more advanced obstructive symptoms, including perforation. Right sided lesions present more insidiously, as the caecum and ascending colon have a larger lumen with more liquid stools passing through. This means that obstructive symptoms are rarer and later. These patients usually present with symptomatic anaemia, weight loss and, potentially, an abdominal mass (72).

Investigation of a patient with suspected colorectal cancer is based around the patient's history, physical examination findings (including rectal examination), laboratory blood test findings and endoscopic examination of the colon with a colonoscopy, including biopsy specimens from any lesions identified as suspicious for CRC. Patients may also undergo a Computerised Tomography (CT) scan of the abdomen as an initial investigation, or, alternatively, a staging CT scan of their whole body as part of their assessment once a CRC lesion has been detected. Other potential staging investigations include, a Magnetic Resonance Imaging

(MRI) scan of the liver, to assess for potential metastases, a MRI scan of the rectum for rectal cancers, to allow better delineation of the anatomy pre surgery, or a trans rectal ultrasound scan (USS), again, to better assess a rectal cancer before deciding on the surgical treatment of the CRC. For those patients who are not suitable to undergo a colonoscopy, then a "virtual colonoscopy", or CT colonography, can be performed to assess for possible colonic tumours (72).

The histological confirmation of CRC is a vital part of the Multi-Disciplinary Team (MDT) approach to the management of patients with the disease. More than 90% of CRC are adenocarcinomas, which originate from the epithelial cells of the colorectal mucosa. This tumour type is characterised by its glandular appearance. It is held that in well differentiated adenocarcinoma >95% of the tumour is gland forming, moderately differentiated adenocarcinoma shows 50-95% gland formation, and poorly differentiated adenocarcinoma is mostly solid, with <50% gland formation. The commonest subtype is moderately differentiated, comprising approximately 70% of CRC adenocarcinoma. Within the bracket of adenocarcinoma, there are some rare histological subtypes, including mucinous, signet ring cell, and medullary type. Other rarer forms of CRC include, neuroendocrine tumours, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas (73).

The staging of CRC tumours is by far the most important prognostic predictor of clinical outcome for these patients. CRC is staged via the TNM system which was first introduced in 1946 (74), and defines the pattern of CRC disease in terms of

depth of tumour invasion (T), the extent of nodal metastases (N), and the presence of any distant metastases (M) (see table 2.3.1 for full TNM staging). This system is utilised, along with the Duke's staging system, which provides a simplified form of staging (see table 2.3.1 for full Duke's staging). Duke's staging was first described in 1929, and, after several revisions, is now well-defined. It describes the tumour pattern in terms of 4 stages, ranging from minimal colonic wall invasion, to more extensive wall invasion, the invasion of surrounding lymph nodes and finally, to include distant metastases (75). The more advanced the disease is, unsurprisingly, the poorer the expected prognosis. Data from 2002 – 2006 in the UK showed that Duke's stage A carries a 5 year survival of >90%, Duke's B >80%, Duke's C >60% and Duke's D 5-10% (8).

TMN staging	
Primary Tumour (T)	
TX	Primary Tumour cannot be evaluated
T0	No evidence of primary tumour
Tis	Carcinoma in situ. Tumour confined to mucosa
T1	Tumour invades the submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades sub-serosa or beyond
T4	Tumour invades adjacent organs or perforates the visceral peritoneum
Regional Lymph Nodes (N)	
NX	Regional Lymph nodes cannot be evaluated
N0	No regional lymph node involvement
N1	Metastasis to 1 to 3 regional lymph nodes
N2	Metastasis to 4 or more regional lymph nodes
Distant Metastasis (M)	
MX	Distant metastasis cannot be evaluated
M0	No distant metastasis
M1	Distant metastasis is present

Table 2.1. Full TNM staging criteria (74).

Dukes Stage		TNM equivalent
A	Tumour limited to submucosa	T1-2,N0,M0
B	Tumour involves muscularis propria	T3,N0,M0
C1	Tumour spread to 1-4 local lymph nodes	T1-2,N1,M0
C2	Tumour spread to more than 4 regional lymph nodes	T3-4,N1-2,M0
D	Distant metastasis	T1-4,N1-2,M1

Table 2.2. Dukes staging and equivalent TNM stage (8).

2.1.4. Treatment

Once CRC has been adequately staged, the treatment is determined by the disease extent. Treatment options include surgical resection of the affected section of the bowel. The type of resection is determined by factors including tumour location, the functional status of the patient, their preference, and the skill of the surgeon. Wherever possible a direct anastomosis is attempted to ensure continuity of the bowel. In cases where this is not technically possible, a stoma, either temporary or permanent, will be sited. However, in patients with low positioned rectal tumours, the patient may have to undergo an abdominoperineal resection and have a permanent colostomy. Previously metastatic disease was considered a contraindication to surgery, however, increasingly; amenable solitary liver or lung metastases are resected either synchronously, or at a later date (72).

Following surgical resection, the cancer will be fully staged by histological examination of the excised tumour. This will allow determination of the depth of tumour invasion, the presence of surrounding lymph node involvement, and any vascular invasion. Patients with Duke's C and D, and some with Duke's B, will undergo adjuvant chemotherapy to reduce the risk of CRC recurrence. Chemotherapy regimens include combinations of Fluorouracil, oxaliplatin and capecitabine. They are given as combination therapy or as a monotherapy, and the choice depends on the patients' functional status, the CRC phenotype, and the oncologist's choice and experience. Some patients with particularly bulky disease may undergo neo-adjuvant chemotherapy, that is, pre-operative chemotherapy,

in an attempt to reduce the size of the tumour, thus possibly allowing easier and potentially curative surgical resection.

In addition, some patients with rectal cancer will undergo neo-adjuvant, or adjuvant, radiotherapy, either to reduce the size of tumour pre-operatively, or reduce the risk of tumour recurrence post-treatment. This may be used in isolation or in conjunction with chemotherapy.

Approximately two thirds of patients present with potentially curable disease, and of these 30-50% will develop recurrent disease (76, 77). Disease recurrence is typically at the site of resection, or the liver or lungs, with 80% of recurrences occurring within 3 years, and most within 5 years (78, 79).

After surgical resection, patients will be monitored for several years to assess for disease recurrence. There is little evidence to suggest that intensive follow-up increases survival, but neither is there evidence to the contrary (80). There is also variation between centres in terms of the form of follow-up, but most patients will undergo a CT scan to assess for potential liver metastases, and a colonoscopy to assess for metachronous lesions (80). The current British Society of Gastroenterology guidelines state that colonoscopy follow-up should be performed 5 years after surgery, and then every 5 years subsequently until benefit is outweighed by co-morbidities (80). However, many centres instead follow the National Comprehensive Cancer Network guidelines, which include a colonoscopy

1 year post-surgery, an annual CT scan for 3 years and 6-monthly Carcino Embryonic Antigen (CEA) monitoring for 5 years (81).

2.1.5. Bowel Cancer Screening Programme:

2.1.5.1 Background

CRC is a condition which is well-suited to screening. It is common, with a high mortality rate, and the symptoms are frequently non-specific and perceived as common, leading to many patients ignoring them. This leads to the disease often presenting at a later and hence more likely incurable, stage. As discussed earlier, prognosis in CRC is strongly linked to stage at diagnosis. Earlier detection of CRC may lead to reduced mortality, with clinically incurable disease being potentially curable if identified earlier. Screening for CRC could also prevent many future cases of the disease by virtue of the detection, and removal, of potentially pre-malignant adenomatous polyps. The natural history of colonic adenomas is not fully understood, but it is believed that the transformation from adenoma to carcinoma takes approximately 10 years. If these lesions are detected early and removed, then the incidence of CRC could be reduced (82).

It has been demonstrated that screening for CRC using guaiac based faecal occult blood tests (gFOBT) can reduce mortality by 16% in people offered screening, and 25% in those who accept screening. Economic analysis of screening CRC has shown cost effectiveness, with a cost per quality adjusted life year gained of <£3000 for gFOBT screening (83).

Screening programmes have been introduced successfully in many countries, including the United Kingdom, which began its programme in July 2006 in England. It was based on biannual gFOBT screening of 60-69 year olds and was subsequently rolled-out countrywide and then to the rest of the United Kingdom, with complete roll-out by January 2010 (83). The upper age limit was then extended to 74 years in 2012, although, those aged over 74 can still request a screening kit to be sent to them. If the kit returns a positive result then the individual is invited to undergo a colonoscopy. There are three main outcomes: 1) if the colonoscopy is normal, then they are discharged from that round of screening and will be invited to take part in the next round 2 years later. 2) If any polyps are detected then the patient will leave the screening programme and enter into the polyp surveillance programme, which is based on the current BSG guidelines for polyp surveillance. 3) If cancer is detected, the patient will follow the MDT route of management for their CRC (83).

2.1.5.2. Effectiveness

Reports into the effectiveness of the Bowel cancer Screening Programme (BCSP) found that uptake of the programme varied according to geographic location, and socioeconomic status. However, across England, uptake of gFOB testing was 52%, which is comparable with pilot studies carried out in the 1980s and in 2000 (83). Women were more likely to return the kit (54.4%), compared to men (49.6%). Abnormal results were found in 2% of all cases, with 2.5% of men returning a positive result – as might be expected, given the higher disease burden in males.

Of those with an abnormal gFOBT; 10.1% were subsequently diagnosed with CRC (11.6% men, 7.8% women), a further 12% of men and 6.2% of women were found to have polyps defined as high risk, and 19.3% of men and 14.6% of women were found to have intermediate risk polyps (83).

The location of CRC detected in the screening programme showed that 28.7% were reported as rectal cancers, and, overall, 77.3% were recorded as left-sided, with only 14.3% recorded as right-sided. This was a slightly surprising finding as it was previously found that the only 66% of CRC was left-sided in non-screening CRC population. 71.3% of detected CRC was found to be the earlier Dukes Stages of A or B, which is again comparable to the 2000 English pilot study (72%) (83).

2.1.5.3. Future

Following the successful extension of the BCSP to 60-74 year olds, a further screening programme, Bowel Scope Screening (BSS), underwent pilot studies beginning in early 2013. This involved a one-off flexible sigmoidoscopy for those aged 55-60 years. At the age of 60, they would then enter the BCSP. Analysis of the 6 pilot centres results showed an uptake of 43.1%, and surprisingly, compared to the BCSP, uptake was higher in men than in women (45% vs 42%). National roll-out of the programme began following these pilots and, as of March 2015, two-thirds of BCSP centres now offer BSS. One of the pilot studies reported they found adenomas in 9.8% of screened patients, and CRC in <1% (84).

Another potential development in the BCSP is the replacement of gFOBT with faecal Immunohistochemical Tests (FIT). This is based around the apparent superiority of FIT testing to gFOBT in terms of diagnostic accuracy, but also with regards to uptake rate (85-87). gFOBT is based on a peroxidase reaction which makes it susceptible to false positive results, especially with diets rich in meat, vegetable and fruit products which contain peroxidase (88). This potentially explains the low positive predictive value of ~10%. FIT testing, meanwhile, is based on antibody testing against human haemoglobin, which allows for quantitative results of the levels of haemoglobin (7). The Scottish BCSP has introduced a two-tier system of gFOBT and FIT (89). A subsequent trial of FIT as the first line screening test revealed a higher uptake rate of 58.5% compared to gFOBT, but a similar positive predictive value, though given the increased uptake this may represent more cases diagnosed (86). Other studies have shown a 13-15% higher participation rate as a result of FIT testing (90, 91). It seems likely that in the coming years FIT testing will replace gFOBT as the screening test for the BCSP.

Other, more novel technologies which are being developed include, Faecal DNA testing, faecal protein testing and pyruvate kinase isoenzyme type M2 (M2-PK).

Faecal DNA testing involves looking for specific genetic changes, such as mutations in the WNT and MAPK pathway genes (e.g. Kras and APC), which are known to be present in neoplastic cells and are shed into the colonic lumen. These tests are in the development phase, and are mostly being used on patients who have been demonstrated to have CRC, and would need assessing in the screening population.

However, they appear promising, with a sensitivity of >85% for CRC and >50% for large adenomas (90). A multi-target faecal DNA test was recently compared to FIT in an average risk screening population, and found to have a sensitivity of 92.3% for CRC, and 42.4% for adenomas with high grade dysplasia. The sensitivity was approximately 20% higher than the comparator; FIT. The specificity was lower for the faecal stool analysis, 86.6%, compared to 94.9% for FIT (92).

Several faecal proteins, such as lactoferrin, lysozyme and albumin have been assessed as potential faecal biomarkers of organic pathology, although, only faecal calprotectin (FC) has shown any promise, due to the poor diagnostic accuracy of the other tests (88, 93). Faecal calprotectin is an inflammatory protein released from white blood cells in response to inflammation or malignancy. It has a mean sensitivity of 83% and specificity of 84% for organic bowel pathology, but, as it is unable to distinguish between malignancy and inflammation, it is unsuitable for CRC screening (88).

Pyruvate kinase isoenzyme type M2 has shown promise as a potential stool biomarker for GI cancers, including CRC. The rationale behind its use is that different isoenzymes of pyruvate kinase are expressed depending on the metabolic functions of tissues. During rapid cellular division, as seen in CRC, specific isoenzymes are replaced with M2-PK in its dimeric form. The main advantages are that only one stool test is required, and points of care tests are available commercially. A pooled analysis of 12 studies, revealed a sensitivity of ~80% for CRC, and 44% for adenomas >1cm. However, this only included 704

patients, and therefore, larger scale studies are required before M2-PK could be used as a screening tool for CRC (88, 94).

Blood serum markers for CRC are also undergoing investigation at present. DNA originating from cancer cells has been found circulating in plasma, which could allow serological screening for CRC. DNA methylation occurs early in carcinogenesis and thus, biomarkers of these epigenetic events may permit earlier diagnosis. Some genes are more heavily methylated e.g. Septin-9 (SEPT9), and this target is currently under assessment in the screening population (90). A commercially available test, including molecular assays for aberrant methylation of BMP3, NDRG4, KRAS and an immunochemical FOB test, is available. This test gives better sensitivity than FOB, but at the expense of specificity (88). The current cost of these DNA sequencing technologies, at present, prevents their use as a viable screening test at present.

There is an ongoing quest for non-invasive biomarkers of disease, particularly cancer. This search has led to the exploration of volatile organic compounds as a potential biomarker.

2.2. Volatile Organic Compounds

The quest for a non-invasive method of screening for, or detecting, early stage cancers has been applied to genomics, proteomics and metabolomics. One of the most promising metabolomic approaches is the detection and analysis of volatile organic compounds (VOCs). The detection of patterns of VOCs by both invasive and non-invasive methods, and their utility as disease specific gas phase biomarkers, has been a rapidly developing area within several medical domains over recent years (95).

VOCs are a widely diverse group of carbon based chemicals and are classified according to their boiling points and retention times. They represent the product of metabolic processes within the body and are simple chemicals such as alcohols, aldehydes, alkanes etc. They are present in exhaled breath as well as blood, urine, faeces and sweat. Alterations in the patterns of VOCs have been suggested to result from pathological processes in the body generating new VOCs, which are not produced during normal physiological processes, thus allowing their use as biomarkers of disease (95).

VOCs have been shown to produce disease specific patterns which allow the distinction of cancer from non-cancer, but also, other non-cancerous diseases from controls, across a wide range of medical specialties, including respiratory; gastroenterology and metabolic medicine (96). The study of this technology has developed as a result of the desire for non-invasive, rapid, point-of-care tests

which will allow patients to be tested for various disease states, and responses to treatment.

2.2.1. Background and Animal studies

Initial interest in non-invasive VOC detection as screening tests for disease arose from the observation of canine olfactory detection of cancer in humans. This was first reported in 1989, with a dog reported to have persistently sniffed a spot on its owners leg which, on medical examination and biopsy, was proven to be melanoma (97). A study in 2004, using 2 separate dogs on 7 patients, found the dogs were able to detect biopsy proven melanoma in 6 of them (98). Another study in 2004, demonstrated that canines were able to detect malignancy in the urine of patients with bladder cancer (99). Further early studies, looking at lung, breast, prostate and ovarian cancer, have all suggested a role for canine detection of human malignancy (100-102). These studies showed varying success rates, sensitivities and specificities. Gordon et al demonstrated only 22% sensitivity for detecting breast cancer in urine specimens, and only a 17% success rate for prostate cancer. The authors suggest that this may be due to the urine being dried first, resulting in evaporation of VOCs, as fresh samples demonstrated a 50% sensitivity (101). Other studies into breast and lung cancer detection via exhaled breath, showed sensitivities of 88% and 99% respectively, and specificities of 98% and 99% respectively (100). A review of the early studies highlighted several problems in terms of lack of controls, and the use of canines, which need to be specifically trained to perform the task (103). Subsequently, a study in 2011, into

canine detection of CRC using both breath and stool samples showed high sensitivity and specificity for both sample types (sensitivity 91%, specificity 99% for breath and sensitivity 97%, specificity 99% for stool). Interestingly, the canine detection remained high even for earlier stages of CRC. This suggests that the changes in the VOC profile occur early in the carcinogenesis pathway. The authors also found that canine scent detection was not confounded by smoking status, the presence of benign polyps or IBD (104).

A further study demonstrated that four trained dogs were able to distinguish bladder cancer from controls through urine, with a pooled sensitivity of 64%, and specificity ranging from 56% to 92%. Again, this study demonstrated no confounding by smoking, gender or age. Although they did find that sensitivity and specificity were affected by other, non-cancerous, urological co-morbidities (105). Better success appears to have been found with prostate cancer. One study reported that using urine specimens, the dog, which had received 24 months of training, was able to distinguish prostate cancer specimens from controls with a sensitivity and specificity of 91% (106). This was followed by a larger study looking at urine from 362 patients with prostate cancer and 540 healthy controls, which found that 2 dogs achieved respective sensitivities of 100% and 98.6%, and specificities of 98.7% and 97.6% (107).

In recent years, subsequent studies into the animal detection of various cancers by bodily substance olfaction have included; studies assessing lung cancer detection from breath samples (sensitivity 71-82%; specificity 82-93%) (108, 109)

and one study assessing lung cancer detection from breath and urine samples (110).

The problems with animal olfactory detection of cancer include; training time for the animal, the variability of training and also 'olfactory fatigue'. This phenomenon describes where the olfactory receptors become saturated with a particular odour and, hence, will lose sensitivity with continuous exposure (111, 112). This, in part, has accelerated the quest to find a technology which will allow for rapid, reproducible testing of bodily samples for VOC patterns that show a high diagnostic accuracy.

Interestingly, as previously mentioned, canine cancer detection has repeatedly been shown to be unaffected by confounders such as smoking status, food odours and drug metabolites. This suggests that while the robotic VOC detection methods may be more reproducible, they lack the "canine element" of being able to tune out potential confounding smells (113). The argued benefits of canine detection over mechanical detection are that dogs give a clear binary response (yes/no) to the presence of cancer, and the sample can be analysed at the bedside, which reduces the risk of sample degradation due to storage (114). Most mechanical forms of VOC detection, with some exceptions, detect individual VOCs, whereas canines are most likely sensing the overall "smell print" of multiple VOCs in the bodily sample.

2.2.2. Current and future technologies and VOC sample type

There are several available analytical tools which can be used to detect VOCs in various bodily substances. The current gold standard is widely held to be GC-MS.

A systematic review from 2015 looked at the use of exhaled breath in detection of all types of cancer. Of these studies, 42 relied on GC-MS as their analytical machine, whilst 24 utilised E-nose technology. A summary of the various technologies used for VOC detection can be found in Table 2.3.

GC-MS technology allows identification of individual VOCs within the specimen chosen for sampling. Significant differences in the type of VOCs found between cancer patients and healthy controls have been identified. Individual VOCs identified vary according to cancer type, however, some have been found to be elevated in cancer compared to non-cancer patients. These include 2-methyl-3phenyl-2propenal; p-cymene, anisole, 4-methyl-phenol and 1,2-dihydro-1, 1,6-trimethyl-napthalene, whereas dimethyl sulphide was found to be present at lower concentrations in cancer patients as compared to non-cancer (115).

Though GC-MS allows identification of individual VOCs, it is a large, expensive piece of equipment, with significant operational costs and specific laboratory requirements. Furthermore, there have been to date no unique VOCs identified which are consistently present through out cancer patients, rather a panel of VOCs are used to identify the cancer patients. These factors mean it is unlikely to translate from research to clinical application easily.

Technology	Benefits	Limitations
GC-MS	• Identify individual VOCs • Most studied/well characterised • Currently held Gold standard	• Expensive • Requires Laboratory infrastructure • Unlikely to be used in clinical settings • Individual VOCs unlikely to be successful as biomarkers
E-Nose	• Can be used at point of care • Recognises overall pattern of VOCs	• Does not identify individual chemicals • Affected by high levels of water vapour • No absolute calibration • Sensors can drift • Not quantitative data
FAIMS	• Can be used at point of care • Recognises overall patterns of VOCs • Rapid sample analysis	• Does not identify individual chemicals • Affected by high levels of water vapour • No absolute calibration • Sensors can drift • Not quantitative data
Other MS based, inc SIFT-MS, TOF-MS, PTFR-MS	• Identify individual VOCs	• Expensive • Requires Laboratory infrastructure • Unlikely to be used in clinical settings • Individual VOCs unlikely to be successful as biomarkers • Limited data/studies

Table 2.3. Summary of the benefits and limitations of the various technologies which are currently being used for detection of cancers by VOC analysis

Miniaturised GC equipment has been under development since the 1970s but, so far has met with limited commercial success, due to combined difficulties in creating stable coatings and poor compatibility with existing GC equipment (96).

E-nose is a broad ranging term which does not describe individual sensor technology but rather the method of detection. In contrast to GC-MS, these sensors do not detect individual chemicals but rather the overall pattern, or "smell print", of the sums of the individual VOCS present, much like a human or canine nose would. The E-noses, typically comprise a sensor array of between 8 and 32 different chemical sensors, which are broadly tuned to different chemical groups including alcohols, ketones and low pressure gases. When the sensor array is exposed to the air above the biological sample, the "headspace", each sensor produces a unique response to the chemicals present. It is possible to extract a feature from this response and use it to train a pattern recognition engine within the machine, and, if presented with a similar VOC pattern in the future, the E-nose will be able to recognise the sample as a specific disease (96).

The types of sensors used for E-noses are wide ranging and include; carbon black composite polymers, semi-conducting metal oxide chemo resistors, polymer coated quartz crystal microbalances, optical dyes and electrochemical sensors. Drawbacks to the E-nose technology include; loss of sensitivity in the presence of high levels of water vapour or single components; the sensors can drift; there is no absolute calibration method and, as previously discussed, the data obtained is not quantitative (116).

In addition to the above technologies, there are now many new technologies which can be included under the E-nose umbrella. These include IMS, GC technology utilising gas sensors as the detector, and optical gas spectrometers. The added benefits of these newer technologies is a more rapid sample processing time of just a few minutes, giving much faster results and potentially allowing for point of care bedside testing of samples (96). Field Asymmetric Ion Mobility Spectrometry (FAIMS), a type of IMS, in particular, appears to give higher sensitivities than the other technologies. It identifies VOCs by tracking the mobility of single ions as they pass through an electric field. Thus, allowing identification of minute changes in the VOC composition, although again, without the ability to detect individual VOCs (96).

Other technologies include Selected Ion Flow Tube Mass Spectrometry (SIFT-MS), which combines chemical ionisation with mass spectrometry. It allows rapid quantification of trace VOCs, even when there is a large amount of atmospheric gases present. Unfortunately, the time for sample analysis is limited to the exhalation time and, so, precise and sensitive quantifications of low concentration compounds could be limited (117). This technology, being based on mass spectrometry, requires laboratory infrastructure and is unlikely to be able to be utilised in clinical settings. Yet, there may be a role for it in the detection of diseases via breath samples, given its ability to detect VOCs in the presence of atmospheric gases. Proton Transfer Reaction Mass Spectrometry (PTR-MS) has also been applied as a potential analytical tool, but it is less accurate than GC-MS,

in that it is unable to distinguish between two compounds of the same molecular weight, although, it is the most sensitive equipment for detecting aromatic hydrocarbons (95).

Potential future technologies include the advent of portable, hand held, E-nose type equipment. Some are already near to commercialisation for non-malignant conditions including; tuberculosis, Clostridium difficile and bacterial overgrowth. The future use of such technology for cancer detection could include the integration of the sensors, with the electronic interface and analytical centre, onto a single chip which could be placed inside a portable device, allowing point-of-care testing. This could revolutionise the clinical screening of conditions such as CRC (96).

2.2.3. Mechanical VOC detection

2.2.3.1. Initial progress

Despite most of the initial interest in the non-invasive detection of cancer having originated from observations of canine detection of cancer, the first studies into the role of VOCs in human cancer detection actually predate the 1989 case report by Williams. As early as 1985, exhaled breath analysis using Gas Chromatography and Mass Spectrometry (GC-MS) was shown to be able to distinguish patients with biopsy proven lung cancer from healthy controls with a classification accuracy of 93%. Using the GC-MS technology they were able to find several chemical peaks which seemed to be unique to the lung cancer patients, although not universally so, meaning that a panel approach had to be used to reach the achieved classification accuracy (118). This was followed in 1988, with an analysis of the components of exhaled gas using GC-MS, which showed that specific chemicals, namely o-toluidine and aniline, were present in the breath of lung cancer patients, but not the control samples (119). Again, anilidine, was not found in all of the patient's breath samples. Subsequently; several potential VOCs, principally alkane and benzene derivatives, were identified as being unique biomarkers for lung cancer (120). The next study to assess the utility of exhaled VOCs as a biomarker for lung cancer was by Phillips and colleagues in 1999. Breath samples of patients with abnormal chest radiographs due to undergo bronchoscopy were collected and analysed using GC-MS. They found that a panel of 22 exhaled VOCs, predominantly alkanes, alkane derivatives and benzene derivatives, discriminated

between those who had benign disease and those patients diagnosed with lung cancer, with 100% sensitivity and 81.3% specificity (121).

Following this research, there was a marked increase the in the number of studies conducted into the non-invasive detection of many different cancers.

2.2.3.2. Lung Cancer

Following the initial efforts of Gordon and Phillips described above, the first decade of the 21st century saw a rapid expansion in the number of studies aimed at analysing the VOC patterns in bodily substances of patients with lung cancer. Krilaviciute et al conducted a systematic review in 2015, analysing the use of exhaled breath in all cancer detection, not just lung cancer. They found that over 73 studies researched exhaled breath VOC detection and, of these, two thirds were focused on lung cancer (122).

Further to their work in 1999, Phillips et al, in 2003, analysed the breath samples of 108 subjects (67 lung cancer, 41 healthy controls) using GC-MS to detect for the presence of a panel of 9 VOCs. They found a sensitivity of 89.6% and specificity of 82.9% (123). Subsequently, they conducted a larger study of 193 lung cancer patients and 211 healthy controls, again using GC-MS to detect a panel of 16 VOCs. They achieved a sensitivity of 84.6% and specificity of 80% (124). This data was later reanalysed using a weighted digital analysis model, giving a slightly higher specificity of 81% (125). There have been several studies which have looked at the ability of GC-MS technology to distinguish or detect lung cancer from exhaled

breath VOCs, including both single and multiple VOCs. The sensitivities and specificities from these studies ranged from 51% - 100%, and 69% to 100%, respectively. To date, no one single VOC has been shown to be consistently effective at distinguishing lung cancer from controls. This suggests that it is the overall pattern of VOCs, which allows distinction of cancer from controls, rather than one single biomarker.

More recently, fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR-MS) has been used to analyse exhaled breath samples from lung cancer patients (126). This has been shown to distinguish patients with lung cancer from ex-smokers, current smokers and patients with benign lung nodules, with classification accuracy of 97%, 95% and 89% respectively (127).

Attempts have been made to identify individual VOCs from the headspace above lung cancer cell cultures using GC-MS. These have shown an altered VOC expression profile between different lung cancer cell types, although again, no consistent tumour marker has been identified, and there appears to be variable correlation with exhaled VOCs (128-132). A problem identified from using cell line cultures is that, as the cells are not in their native environment, their metabolism will be different and produce a different VOC profile (133). It has also been suggested that cell culture methods do not provide as relatively hypoxic an environment as that found in vivo during carcinogenesis of lung cancer, and that cell culture mediums should attempt to mimic this environment (134).

Technology other than GC-MS has also been utilised. In 2005, Machado et al used a Cyranose 320 Electronic nose (E-nose) to compare exhaled VOCs from 14 lung cancer patients to 20 healthy controls, and create a training set for the machine. Validation analysis of a further 14 lung cancers, with 62 controls, showed that the E-nose could discriminate cancers from controls with a sensitivity of 71.4%, and specificity of 91.9% (135).

Dragonieri then demonstrated that the same E-nose model could distinguish lung cancer patients from those with Chronic Obstructive Pulmonary Disease (COPD) and healthy controls with an accuracy of 85% and 90%, respectively, from exhaled breath (136). McWilliams et al demonstrated that the Cyranose could distinguish exhaled breath from lung cancer patients from high risk controls (smokers and COPD patients) with an accuracy of >80% (137).

Other types of E-nose technology include colorimetric sensor arrays, which were used by Mazzone et al, in 2007, to study exhaled lung cancer VOCs. A colorimetric sensor array is embedded with chemically sensitive compounds which change colour in response to chemical stimuli. They determined that the array could distinguish lung cancer patients from other pulmonary conditions, including pulmonary fibrosis, COPD, sarcoidosis and also healthy subjects, with a sensitivity of 72.3% and specificity of 72.4% (138). In 2012, they augmented their protocol by using clinical data such as smoking status, age and sex to improve the accuracy of their model with an area under the curve (AUC) of 0.811. It also demonstrated some promise in distinguishing squamous cell cancers from adenocarcinomas

(AUC 0.864), early from late stage disease (AUC 0.784) and survival <12 months vs survival >12 months (AUC 0.770) (139). The same group further refined their technique and were able to achieve accuracies of 79% to 86% for distinguishing cancer patients, and subgroups, from controls (140).

Peng et al used GC-MS to identify potential VOCs from breath samples from multiple cancer types and then trained a gold particle based nano-sensor array (a variant of an electronic nose). They were able to distinguish lung cancer patients, from those with CRC, prostate cancer, and healthy controls (141). Barash et al also used a gold nano-particle array to distinguish between the breath of non-small cell lung cancer patients and controls with 100% accuracy (142).

In 2003, Di Natale et al used a Quartz sensor based E-nose and demonstrated 90.3% accuracy for discriminating between the breath of lung cancer patients, healthy volunteers and post-surgery lung cancer patients (143). The same group carried out a further study in 2009, and showed that the exhaled breath of confirmed lung cancer patients could be distinguished from healthy non-smokers with a sensitivity of 85% and specificity of 100%. It could also distinguish lung cancer from other lung diseases with a sensitivity of 93% and specificity of 79% (144). Santanico et al also used a Quartz sensor based E-nose to compare 20 confirmed lung cancers with 10 healthy controls. They analysed exhaled breath with bronchoscopically obtained alveolar air and found that the bronchoscopically obtained sample gave better results than the exhaled samples, with a sensitivity of 97.5% and specificity of 75%. They also found a 75% classification accuracy

between squamous cell cancer and adenocarcinoma patients (145). Recently, further attempts to use Quartz sensor based E-nose technology has looked at distinguishing lung cancer patients from healthy controls in the presence of potential metabolic confounders such as diabetes, obesity and dyslipidaemia. This found an overall sensitivity and specificity of 81% and 91%, respectively. Comparing the presence of metabolic confounders, against their absence, gave a sensitivity and specificity of 85% and 88% vs 76% and 94%. They also found that when analysed by stage of lung cancer, the best sensitivity was for stage I (92%) vs stage II/III/IV (54%) (146).

In 2009, Westhoff et al, used a Nickel-63 based Ion Mobility Spectrometry (IMS) device to analyse exhaled breath of lung cancer patients. IMS technology separates VOCs by virtue of their mobility in response to being passed through an electrically charged field. Westhoff et al were able to distinguish between 32 lung cancer patients and 54 healthy control subjects, including smokers and non-smokers, with an accuracy of 100% (147). Subsequently IMS technology has been used to demonstrate that the VOC profiles of patients with lung cancer vary between different histological subtypes of tumour, and between the diseased and disease-free lung (148).

Peled et al used a custom made sensor array combining carbon nanotube technology with gold nano-particles to analyse exhaled breath VOCs. This allowed differentitation between cancerous and non-cancerous pulmonary nodules with an accuracy of 88% (AUC 0.986). It was also able to distinguish early from late

disease, and adenocarcinoma from squamous cell carcinoma with accuracies of 88% and AUCs of 0.961 and 0.974 respectively (149).

There have been suggestions that VOCs could be used in the follow-up of patients undergoing treatment, including curative resection for lung cancer, similar to the use of tumour markers for monitoring response to treatment and for potential disease recurrence. Poli et al analysed exhaled breath at one month, and at three years post lung cancer resection. They demonstrated that at one month, the only VOC to change in level was isoprene. At three years, some VOC levels, for example, pentane, had increased and some, such as isoprene, had decreased. It was found that most VOCs in the post-surgical samples were still present at higher levels than controls. This suggests there may be a potential role for the use of VOCs in post cancer treatment monitoring, but that further work is required to determine exactly how the VOC profiles change in response to treatments over time (150).

More recently, E-nose technology (Quartz sensor based) was compared to GC-MS in the detection of lung cancer via analysis of breath samples from both the affected and unaffected lung. It was found that the E-nose gave a classification accuracy of over 90%, regardless of the lung from which the sample was taken, whilst the GC-MS only gave a 76% correct classification when both lung samples were compared. Interestingly, they found that the VOC profile was preserved between bronchoscopically sampled air from the lung and exhaled breath (151).

Other E-nose technology, BIONOTE, has been used to distinguish lung cancer patients from healthy controls with a sensitivity and specificity of 86% and 95%, respectively (152).

Other than the main histological types of lung cancer, VOC detection has also been used to study malignant mesothelioma. In 2012, Dragonieri's et al demonstrated that E-nose could distinguish patients with malignant pulmonary mesothelioma from patients with asbestosis with a sensitivity of 92.3% and specificity of 85.7%, from exhaled breath (153). Chapman et al have also demonstrated 85% accuracy in detecting mesothelioma from patients exhaled breath using E-nose technology (154).

Whilst most studies involving lung cancer VOCs have been based around patients' exhaled VOCs, some have looked at other sources. VOCs derived from pleural fluid aspirates have also been studied to distinguish malignant effusions from benign effusions using GC-MS technology, with some promise in terms of unique VOC profiles (155). Urinary VOCs have been studied using GC-MS analysis and 9 potential VOCs were shown to have individual sensitivities and specificities ranging 85-95% and 70-100% respectively, with AUCs of 0.79 - 0.96 (156).

2.2.3.3. Gastrointestinal Cancer

After lung cancer, the GI tract has been the main system studied for the non-invasive detection of cancer via VOC detection, with the bulk of the studies focusing on CRC.

The majority of the research into CRC detection through VOC analysis has looked at patients exhaled breath and urine samples, mainly using GC-MS technology for analysis, with E-nose being the next most commonly used method (157).

Peng et al, appear to be the first group to demonstrate that CRC could be detected by VOC analysis, in 2010. Using a custom-made gold nanoparticle E-nose sensor array and GC-MS to analyse exhaled breath samples, they were able to prove that they could distinguish patients with CRC, lung cancer, breast cancer and prostate cancer from each other, and from healthy controls (141). The E-nose technology demonstrated that 26 CRC patients were completely distinguishable from 22 healthy controls. Although, the sensitivity dropped when GC-MS analysis of 6 identified VOCs were used. This would suggest that it is the overall pattern of chemical components, rather than individual chemical compounds, which contribute towards the distinction of the two groups.

Subsequent breath analysis studies have shown further promise for distinguishing CRC patients from healthy controls. Altomare et al, in 2013, analysed 37 CRC patients and 41 healthy controls using GC-MS. By applying a profile of 15 VOCs, they were able to distinguish CRC patients from controls with 86% sensitivity and

83% specificity, with an AUROC of 0.85. They performed a validation study on 25 blinded subjects which showed an accuracy of 76% (158). Wang et al demonstrated that 9 VOCs were significantly higher, and 1 VOC significantly lower, in the exhaled breath of 20 CRC patients, as compared to healthy controls (159). Amal et al analysed the exhaled breath of 65 CRC patients, 22 patients with adenomas and 122 healthy controls, first using GC-MS to identify potential VOCs, and then utilising E-nose technology. From this, they achieved a sensitivity of 85%, specificity of 94% and an accuracy of 91% (160).

The use of urinary VOCs to distinguish CRC subjects from healthy controls has also been studied in this context. This was first done in 2009 by Ma et al, who compared urinary VOCs of 24 CRC patients with 80 healthy volunteers using Time of Flight mass spectrometry (TOF-MS). They were able to distinguish the controls from the CRC patients. They found statistically significant higher levels of 2 low molecular weight compounds in the CRC patients compared to the healthy controls, and also found that, post-treatment, the levels of the 2 chemicals were significantly reduced (161). Silva et al analysed the urinary VOC profile of 12 CRC, 7 lymphoma and 14 leukaemia patients compared to 21 healthy controls. Their conclusion was that a panel of 16 VOCs could distinguish cancer patients from healthy controls; however, this study did not specifically compare CRC with the other cancers (115).

Two subsequent studies into urine were conducted by Arasaradnam et al. The first of these studies utilised FAIMS technology to analyse the urinary VOCs of 83 CRC patients and 50 healthy controls. It achieved 83% sensitivity and 60% specificity in

distinguishing the 2 groups (162). I was second author for this study and this thesis is primarily a continuation of this work and the questions that were raised from it. Subsequently, a bespoke E-nose, the WOLF system, was developed by the University of Warwick engineering department. This system comprised a combination of different sensor types. This was used to analyse the urine of 39 CRC patients, 35 irritable bowel syndrome patients and 18 healthy controls; achieving a sensitivity of 78% and specificity of 79% (161). Neither study identified any unique VOCs when GC-MS analysis was used. This again suggests that it is the overall "smell print" which is important, rather than individual chemical components. The main results of these studies are shown in figures 2.1 and 2.2. The authors considered that since urine samples were far more acceptable to patients than collecting stool samples, that urine use in screening programmes would be likely to improve compliance (163).

VOCs from stool samples were first studied in CRC patients in 2014 by De Meij et al. They used a Cyranose320 E-nose to assess 40 CRC patients, 60 patients with advanced adenomas and 57 healthy controls. They demonstrated sensitivity of 85% and a specificity of 87%, for distinguishing CRC patients from controls. Having also conducted FIT testing on the same samples, they found a sensitivity of 63% and a specificity of 100% (164). Similarly, Batty et al studied the faecal VOCs in 31 CRC/high grade adenoma patients compared to 31 controls, using SIFT-MS, and they demonstrated a sensitivity of 72% and specificity of 78% (165).

To date, there has been one study evaluating the VOC profile of blood from patients with CRC. Wang et al demonstrated that 3 VOCs were significantly less expressed, and 1 was expressed at significantly higher levels in 31 CRC patients as compared to 31 healthy subjects (166).

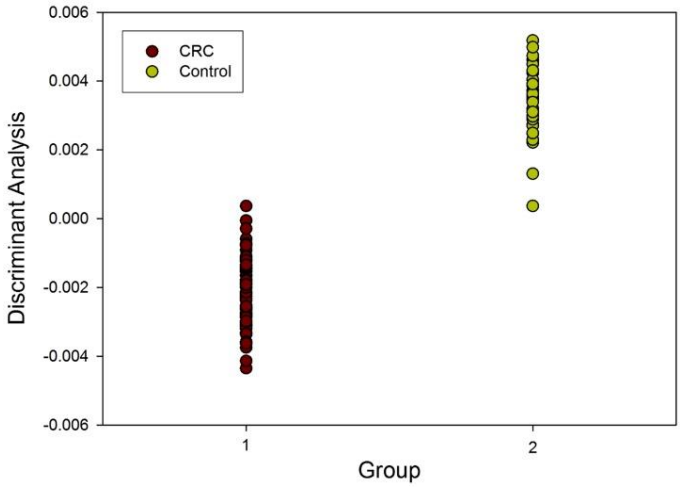


Figure 2.1. Results of Arasaradnam et al, demonstrating distinction of CRC from healthy controls via urinary VOC detection using a FAIMS instrument with 83% sensitivity and 60% specificity (160).

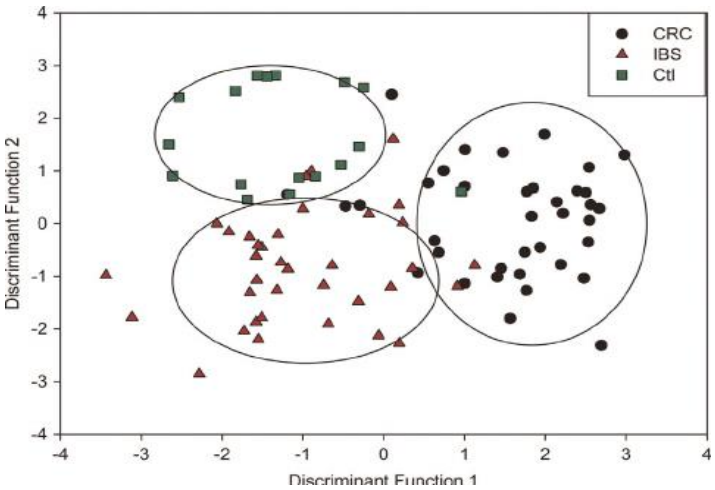


Figure 2.2. Results of Westenbrink et al, demonstrating distinction of CRC from healthy controls and IBS via urinary VOC detection using a custom made E-nose instrument with 78% sensitivity and 79% specificity (161).

The profiling of VOCs to detect upper GI cancers, such as oesophageal, gastric, pancreatic and hepatobiliary cancers has also been studied. Xu et al demonstrated, in 2013, that the exhaled breath of patients with gastric cancer could be distinguished from benign gastric conditions, such as gastritis and ulcers, with a sensitivity of 89% and specificity of 90%, using GC-MS analysis (167). One group has studied the VOC patterns in the exhaled breath, urine and gastric contents of patients with oesophago-gastric cancer using SIFT-MS. Using these VOCs they were able to distinguish the cancer patients with an accuracy of 0.91 in exhaled breath (168), 0.90 in urine (169) and 0.90 in gastric contents (170). Further to this, they carried out a larger validation study using the exhaled breath of 81 upper GI cancer patients and 129 controls, and found an AUC of 0.97 for oesophageal cancers and 0.98 for gastric cancers (171). Amal et al followed up their work on CRC by analysing the exhaled breath of 484 patients, including 99 with gastric cancer, initially using GC-MS to identify potential VOCs, and then utilising cross reactive nano-array E-nose type technology. Using the GC-MS they identified 8 VOCs and with the nano-array, they achieved sensitivity of 73%, specificity of 98% and accuracy of 92% (172).

Two separate studies have used tissue cultures to try to identify potential VOCs in gastric cancer. Buszewski et al compared VOCs from gastric cancer with those of healthy gastric tissue taken from the stomach of the same cancer patients, and found that the cancer cultures produced different VOCs (173). Zhang et al used GC-MS to identify potential VOCs for gastric cancer and then built a Carbon Nano-

tube E-nose array which was able to distinguish gastric cancer cells in culture, from healthy gastric mucosa in culture (174). A more recent study has shown that gastric cancer VOCs appear stable over the course of several days, but can be influenced by changes in the gut microbiome (175).

Attempts to characterise the VOCs of patients with pancreatic and biliary pathology have been made in two studies. Navaneethan et al used SIFT-MS to analyse the VOC profiles in the bile of 24 patients with pancreatic cancer, compared to 72 patients with benign biliary strictures; achieving sensitivity and specificity of 83 and 82% respectively (176). The same group then studied the VOC profiles in urine to try and obtain the same outcome; achieving a sensitivity of 80% and a specificity of 100% (177).

There have been two studies into the VOC profiles of Hepatocellular carcinoma (HCC) patients. This was first done by Qin et al in 2010, using GC-MS to analyse the VOCs in the exhaled breath of patients with HCC, compared to healthy controls and patients with cirrhosis. They determined that a panel of 3 VOCs could distinguish HCC from healthy controls, with a sensitivity of 83.3% and specificity of 91.7% (178). Amal et al then studied the VOC profiles from tissue cultures of HCC cells using GC-MS, and found they could completely distinguish HCC from healthy controls (179).

2.2.3.4. Breast Cancer

Following lung cancer and CRC, breast cancer is the malignancy that has received the most interest and study into non-invasive VOC profiling. Most of the studies to date have revolved around GC-MS analysis of exhaled breath samples, and have been conducted by Phillips et al, who also extensively studied lung cancer VOCs. Their first study, in 2003, assessed a cohort of women with abnormal mammograms, with 51 out of 198 being subsequently diagnosed with breast cancer. GC-MS analysis of exhaled breath samples gave a sensitivity of 88.2% and specificity of 73.8%. They found that breath analysis had a better negative predictive value than screening mammogram (99.93% vs 99.89%), but a weaker positive predictive value (1.29% vs 4.63%) (180). The group then refined their technique and used a panel of five VOCs, resulting in an improvement in sensitivity to 93.8% and specificity to 84.6% (181). Attempts to further refine their analysis in 2010 yielded a slightly poorer sensitivity of 78.5%, but an improved specificity of 88.3% (182). In 2014, the same group studied a point of care breath test, using GC-MS analysis. The breath test had a 6 minute turnaround time. They were able to achieve a sensitivity of 75.8%, specificity of 74% and accuracy of 78% for distinguishing between biopsy proven cancer and biopsy proven non-cancer. Additionally, they found that it could detect breast cancer, where the screening mammogram was normal with a sensitivity of 81.8%, specificity of 70% and an accuracy of 79% (183).

Other studies into breast cancer detection via exhaled VOC detection have been on a smaller scale. Shuster et al studied a cohort of 36 women, with 13 confirmed breast cancer patients. They used GC-MS analysis of exhaled breath and achieved a sensitivity of 94% and specificity of 80% for detecting benign breast disease (184). Patterson et al studied a cohort of 20 breast cancer patients and 20 healthy controls and, using GC-MS analysis of exhaled breath, were able to achieve a sensitivity of 72% and specificity of 64%, with an accuracy of 77% (185). Mangler et al studied a small cohort of 10 breast cancer patients and 10 healthy controls. They found that 4 VOCs were significantly reduced in the breast cancer patients compared to the healthy controls, and that 1 VOC was significantly increased (186).

More recently, a larger study of 276 participants, by Barash et al, attempted to discriminate between patients with malignant breast lumps, ductal carcinoma in situ (DCIS), benign conditions and healthy volunteers, using GC-MS and nano-array analysis of exhaled breath samples. GC-MS identified 23 potential VOCs, and subsequent nano-array analysis gave an accuracy of 83% for distinguishing benign from malignant conditions. Distinction between the various subtypes was achieved with sensitivities ranging from 81-88%, specificities from 76-96% and accuracies of 82-87% (187).

There has been one study, to date, which analysed the urinary VOC profile of 26 patients with breast cancer and 21 healthy controls using GC-MS. There were 6 VOCs identified which had statistically significant levels between the breast cancer

patients and healthy controls. Analysis resulted in an accuracy of 70.3% for discriminating between the two groups (188).

2.2.3.5. Other cancers

Other cancers that have been studied include head and neck, thyroid, ovarian, urological and skin cancers.

Following on from the initial work of Gordon, in 2008, into the canine detection of prostate cancer, and the study of Peng et al comparing lung, breast and prostate cancer in 2010, there have been three studies looking at the detection of urinary VOCs to distinguish prostate cancer patients from healthy controls. Asimakoupolus et al analysed the urinary VOCs of 41 patients undergoing prostate biopsy, using an E-nose. 14 patients went on to have biopsy-proven prostate cancer, and the E-nose was able to distinguish cancer from non-cancer with a sensitivity of 71.4% and specificity of 92.6% (189). Later in 2014, Roine et al compared the urinary VOCs of 50 patients with confirmed prostate cancer, to those with benign prostatic hyperplasia, again using an E-nose. They were able to distinguish between the two groups with a sensitivity of 78% and specificity of 67%, AUC 0.77 (190). Khalid et al analysed the urinary VOCs of 59 prostate cancer patients compared to 43 cancer-free controls with GC-MS; using a model based around a panel of 4 VOCs, and taking into account prostate specific antigen (PSA) levels. They also used several statistical models, and achieved sensitivities ranging from 72-83%, and specificities ranging from 24-58% (191).

The first study to assess the use of VOCs in the detection of ovarian cancer was in 2010, when Horvath et al analysed the VOCs arising from 15 tissue samples of ovarian cancer using an E-nose. They achieved a pooled sensitivity of 84.8% and specificity of 86.8% (116). In 2015, Amal et al, studied the breath samples of 182 subjects, including 48 with known ovarian cancer, other benign ovarian pathology, and healthy controls using GC-MS and a nano-array. They were able to distinguish the cancer patients from the controls with a sensitivity, specificity and accuracy of 71%. The technique was more sensitive for distinguishing cancer patients from healthy controls, achieving a sensitivity of 79%, specificity of 100% and accuracy of 89% (192).

Head and neck cancers have also undergone evaluation for VOC signatures. This was first done in 2008, by Schmutzhard et al, comparing the exhaled breath samples of 22 patients with head and neck squamous cell cancer (HNSCC) to healthy controls, high risk patients and post-therapy patients, using GC-MS. They concluded that there were statistically significant differences between the groups for some VOCs, but did not calculate sensitivities or specificities (193). Leunis et al then followed this up, in 2014, by using an E-nose to compare the exhaled breath of 36 HNSCC patients to 22 patients with benign conditions. They achieved a sensitivity of 90% and specificity of 80% (194). Subsequently, Gruber et al studied 87 participants, including 22 with HNSCC, and 21 with benign conditions, using GC-MS and a nano-array approach. They achieved a sensitivity of 77%, specificity of 90% and overall accuracy of 83% for distinguishing the HNSCC from the healthy

controls (195). HNSCC has more recently been distinguished from lung cancer via exhaled breath analysis using an E-nose, with an accuracy of 85% (196).

VOCs from skin biopsies, analysed by GC-MS, have also been demonstrated to have a distinct profile compared to healthy skin biopsies from the same patients in two studies (197, 198).

2.2.4. VOC generation and detection in bodily secretions

A potential issue with the use of VOCs as a screening tool for the detection of VOCs, is that the precise mechanism of VOC generation has yet to be elucidated. Several theories have been postulated for the mechanism by which VOCs are generated.

It has been proposed that VOCs produced by tumour cells are the product of Major Histocompatibility Complexes (Human Leucocyte Antigen – HLA, in humans) genes within cancerous cells. This theory is supported by the finding that human body odour is genetically determined by HLA. HLA proteins are soluble, and detectable isoforms are present in bodily fluids such as blood, urine and sweat. It has also been demonstrated that there is a strong link between changes in HLA expression and carcinogenesis. Cancer cells are able to evade the host immune system by alterations in the expressions of HLA molecules, in a similar mechanism to that seen by the evasion of maternal immunity in the developing foetus. This suggests that carcinogenesis could result in an alteration of HLA expression in the tumour cells, resulting in a different odour profile of the patient, allowing its detection by VOC analysis (199).

Other theories suggest that VOCs are generated by cancerous cells in response to the overall abnormal cellular metabolism, and alterations in non HLA gene/ protein expression. This is thought to be as a result of carcinogenesis and the activity of reactive oxygen species, which can cause protein oxidation and lipid

peroxidation, thus affecting the VOC profile (200, 201). There is also some evidence that cytochrome p450 enzymes may play a role in the generation of aberrant VOCs, as their activity is elevated in cancerous processes, possibly to neutralise excessive levels of reactive oxygen species and their derivatives (200).

The role of oxidative stress has been questioned by Darwiche et al, who found that bronchoscopically obtained VOC profiles were different between the affected and non-affected lung, in lung cancer patients. They suggested that the VOC generation was more likely due to metabolic tumour processes +/- microbiological activity (148). This finding was supported by Wang C et al, when they extracted breath samples from both the cancer-containing lung and the disease-free lung (201), and by several studies looking at VOC generation in the headspace above lung cancer tissue cells. Chen et al demonstrated, using solid phase microextraction-gas chromatography (SPME-GC), that squamous cell carcinoma, adenocarcinoma, bronchoalveolar carcinoma, non-small cell carcinoma and bronchial epithelial cells all had distinct VOC profiles (202). Other studies have confirmed that lung cancer cells release higher levels of certain metabolites compared to healthy controls, and also that lung cancer tissue appears to have lower levels of certain metabolites. Thus, suggesting they are either not produced, or are in fact consumed by the cancerous cells in their metabolic processes (203, 204).

Other factors which can influence the VOC profile include external elements such as environmental pollution, medications and diet (Peng 2010). In fact, it has been

demonstrated recently that proton pump inhibitors can result in a much altered, and less diverse intestinal flora profile, which would affect any fermentation occurring in the GI tract and, potentially, alter the VOC profile (205). Indeed, a study by Amal et al showed that the VOC profile of gastric cancer patients was stable over repeated sampling, except where interventions which alter the intestinal microbiome have occurred. This resulted in affected VOC profiles, and, as such, anything which affects the microbiome could result in an altered VOC profile (175). This highlights the potential role that the microbiome could have on the overall VOC signature for an individual. Certainly, the current most plausible theory behind VOC generation is that it represents the complex interaction between bodily metabolic processes (including altered disease metabolism), microbiome metabolic processes and dietary factors (96).

VOCs present in exhaled breath can be either exogenous or endogenous. Exogenous VOCs are chemicals which are inhaled from the external environment and then exhaled unchanged. Endogenous VOCs are compounds which are produced within the body as a result of metabolic processes. They can also be compounds produced by the metabolic process of symbiotic bacteria in the human body (206). Hakim et al reviewed all identified exhaled VOCs for lung cancer in 2012, and found that 112 VOCs had been identified from the breath of lung cancer patients and 88 VOCs had been identified from lung derived cancer cell lines. On further analysis, they found that 36 VOCs were identified in two or more studies. These were divided into 7 chemical families: hydrocarbons (e.g. alkanes and

alkenes); primary and secondary alcohols; aldehydyes; ketones; esters; nitriles and aromatic compounds (200).

Breath analysis is the most common means of VOC detection in the literature, and principally, for lung cancer diagnosis. The mechanism by which VOCs are found in breath is believed to be via the release of abnormal VOCs from the tumour into the blood stream, and then subsequent gas exchange in the alveoli of the lung, resulting in their presence in exhaled breath (200). Breath analysis, compared to blood and urine samples, is less invasive and can be collected easily at any point, allowing for simple repeat sampling. Less stringent storage conditions are required, the samples often do not require any form of processing prior to analysis, and VOCs have been found to be present in higher concentrations than in blood samples (95, 200). There are some issues with exhaled breath, these include that approximately only half of the exhaled VOCs are endogenous, and the other half are exogenous. This can present a problem in terms of determining whether a VOC is a biomarker or not. Food consumption, smoking and oral and pharyngeal bacterial colonies can also affect the VOC composition (95). The logistics of breath sampling can also be a challenge. The first part of exhalation is "dead space", that is ~150ml of air which has come from the upper airways, rather than air from the deeper lung airways and alveoli, which would have been involved in gas exchange. The dead space air has little, or no, value in VOC analysis. The best fraction of breath to analyse is that latter part of exhaled breath, or alveolar breath. This will also reduce contamination by exogenous VOCs. It has been

demonstrated that different fractions of breath have affected the ability of a Cyranose E-nose to detect lung cancer (207). There are adaptable device mouth-pieces available which allow the preferential extraction of alveolar breath, without the need to make the patient hyperventilate, but this adds a layer of complexity to the sampling (208). A major problem with all of the studies conducted so far is that there is no consensus on what breath sampling method constitutes a gold standard. Many different breath samples and sampling systems have been utilised, and this often prevents direct comparison between the results of the various studies (209).

The presence of VOCs in various bodily substances is the result of their transport in the blood stream throughout the body. This allows their delivery to the renal glomerulofiltration unit, and thus, their presence in urine, and global delivery to the bodily sweat glands (210).

Sweat VOCs are mainly derived from the secretions of sweat glands, but can also be derived from sebum; the secretions of the sebaceous glands. Although some of the VOCs are the direct result of internal metabolic or hormonal changes, most appear to be produced as a result of skin symbiotic bacterial action on secreted compounds. Any alteration in either the nature of the secreted chemicals or the skin cells themselves, or the bacteria living on the skin, will result in an alteration of the VOC profile (206).

The analysis of blood and urine samples for biomarker VOCs is also not without issue, as the VOCs may not necessarily be endogenous. Exogenous VOCs could be inhaled and bound or dissolved in blood or bodily compartments, then later excreted in urine. They can also be affected by smoking status, medications, diet and other environmental factors. Urine has the advantage over blood that the analytes are concentrated in the kidneys before being excreted, which should allow for easier detection of low concentration chemicals. A summary of the various bodily secretions to contain VOCs and benefits and limitations of each can be found in Table 2.4. Despite these potential confounding factors, there is growing evidence, as described above, that clinically relevant biomarkers can be found in urine, blood, stool and breath of cancer patients (95).

Bodily secretion	Benefits	Limitations
Breath	• Easy to collect • Less invasive • No processing prior to analysis • Acceptable to patients • Most studied secretion	• No concensus on optimal collection method • Contamination by mouth bacteria/flora • Smoking confounding • VOCs for non-respiratory conditions may be at lower concentrations
Urine	• Easy to collect • Less invasive • Acceptable to patients • VOCs at high concentrations due to renal filtration	• Potential contamination from genitourinary bacteria • No consensus on optimal storage • Requires removal of particulates • VOCs may be lost if container not immediately sealed
Faeces	• May have had direct contact with colonic pathology	• Less acceptable to patients • Collection more difficult • Potential microbiome fermentation effects on VOCs from colonic pathology • VOCs may be lost if container not immediately sealed
Blood	• Acceptable to patients	• Invasive • Limited studies
Sweat	• Easy sample collection	• Affected by deoderants, soaps and cosmetics • Limited studies

Table 2.4. Summary of the benefits and limitations of the various bodily secretions currently being studied for the detection of cancers by VOC analysis

2.2.5. Clinical applications - screening, diagnosis, monitoring

As previously discussed, most studies to date have centred on the detection of VOCs in the exhaled breath of patients with lung cancer. The long term goal of this research is to use VOC detection as a non-invasive screening tool, thus allowing more targeted use of CT scans, bronchoscopy and endoscopy.

There have been some promising results for individual biomarkers eg. Hexadecanal, but good levels of discrimination often required the combination of multiple VOCs to form a panel, or "smell print". They also noted reports of the same biomarker being implicated in multiple cancer subtypes e.g. methanal in breast, prostate and bladder cancer. This would suggest that it is of limited value in targetted cancer screening programmes, for example prostate or colorectal cancer, because of the lack of specificity. Additionally, the authors found a huge degree of variability in the methods of breath collection, sample storage and data handling. They recommended larger studies, in true screening settings, using standardised breath collection techniques, with independent validation studies (122).

With regards to the GI tract, breath samples have been shown to be able to detect CRC in patients using GC-MS technology with a sensitivity and specificity of 85%, and may show potential for application as a non-invasive test in the future (158). In addition, early urinary VOC analyses from CRC patients have shown a sensitivity of 83% and specificity of 60% (162). A major fault of most breath sampling studies

so far, as identified by Smith et al in their 2015 discussion paper, is that the majority have collected mouth-exhaled breath, rather than nose-exhaled breath. This leads to contamination by oral cavity generated compounds as a result of bacterial and enzymatic activity (117). At present there is no agreed upon protocol for the ideal collection method for breath samples. Stool samples are likely to have a lower uptake rate, as seen in the current BCSP. Therefore, the utilisation of urine as a potential screening medium for CRC detection may present a more acceptable form of specimen to patients than faeces. The use of urine has other advantages beyond patient acceptance. Due to the renal filtration process, VOCs are present in urine at nearly the same concentrations as in the plasma (115). This suggests that there will be minimal signal lost due to the renal filtration process, although, as yet, no studies have compared the differences in VOC composition or pattern between plasma samples and urine samples for the same patients.

A study into the post-surgical changes of VOCs in lung cancer patients revealed that there were some minor changes at one month – reduced isoprene levels, and some further changes by three years. Most of the VOCs sampled were still present at higher concentrations than in the healthy control group (150). Altomare et al also conducted a post-surgical resection study of exhaled VOCs from CRC patients. They analysed the exhaled VOCs of 32 disease free CRC patients, 2 years post-surgery, and compared their VOC profiles to samples collected pre-operatively. They found a panel of 11 VOCs could be analysed with GC-MS and distinguish pre-operative from post-treatment samples with a sensitivity of 100%, specificity of

97%, accuracy of 98.8% and AUC 1. When comparing the post-surgery VOCs with healthy controls they also found a distinct pattern with a sensitivity of 100%, specificity of 90%, accuracy of 94% and AUC of 0.96 (211). This had previously also been shown by Ma et al, in 2009, as discussed earlier (161). These studies suggest that, whilst the VOC profile does alter post tumour removal, it does not completely revert to what might be expected in the general population. This may indicate that, although cancer metabolism contributes to the generation of VOCs, it may not be the sole factor responsible for the VOC profiles seen in cancer patients. The residual different VOC profiles could be due to changes produced in the microbiome of the subject's lung, either leading to, or as a result of carcinogenesis.

A more recent study has shown that 4 cancer specific VOCs reverted to the levels of healthy control subjects in lung cancer patients who had successfully undergone resection (212). These studies do appear to support the hypothesis that VOC profiling could have a role, not just in the screening or diagnosis of patients for malignancy, but also as an aid in follow-up to assess for disease recurrence, in the same way as other tumour markers, such as CEA, are currently used.

2.3. Microbiome in CRC

As already discussed, risk factors which can predispose to CRC include obesity; dietary factors, including red or processed meat, dairy products, fibre, vitamin D and micronutrients; medications; alcohol and smoking. A western style diet, in particular, that is high in red meat and animal fats, and low in fibre, is associated with an increased risk of CRC development.

There has been rapidly expanding interest over recent years into the role that gut bacteria, or the microbiota, play in the health and disease of humans. There appears to be growing evidence that the development of CRC could be linked to alterations in the gut microbiome. The first study suggesting a link between gut microbiome and CRC was reported in 1975 when germ-free rats were observed to develop fewer chemically induced CRCs than wild-type rats (213). This finding has been subsequently replicated, including in CRC predisposed mice (214).

The human body contains well over 100 trillion ($1x10^{14}$) microbial cells, with the GI tract having, by far, the highest density within the body, at approximately 10 trillion micro-organisms, including bacteria, viruses and fungi, constituting the microbiota (215-217). The composition of the microbiota in different parts of the GI tract appears relatively stable, although the absolute numbers of micro-organisms vary enormously between the mouth and rectum (214).

The gut microbiota in an individual is developed during the first few years of life, and is a result of colonisation from the commensal flora from the mother's skin,

vagina and faeces initially, and then, due to complex interactions between micro-organisms introduced via the environment, and the hosts physiological processes (214). Once the microbiome has stabilised after the first few years of life, the composition remains relatively stable over time, with some fluctuation in response to pathological and environmental factors (214, 218). This stability is thought to represent the long term influence of dietary patterns on the microbiome (219). It is increasingly believed that the development and maturation of a healthy immune system is vitally dependent on the formation of a balanced and diverse gut microbiome in early life. This is based on the observations of immune system abnormalities found in germ-free animals reared in bacteria-free conditions (214).

Evidence for the stability of the gut microbiome being due to dietary factors can be found from studies which show that switching from a diet high in fibre and plant polysaccharides, and low in animal fat and processed sugar, to a reciprocal diet, leads to an altered microbiome composition within one day of the dietary change (218, 220, 221). The alteration in microbiome composition included an increase in bile-tolerant bacteria e.g. Bilophilia and bacteroides, and a decline in bacteria which metabolise plant polysaccharides e.g. Roseburia. These changes would be expected in the face of this dietary alteration and represent a degree of natural selection (220). However, these changes are not sustained, as, on resuming the previous diet, the microbiome reverts to the pre-change composition (219). This would indicate that if CRC development was linked to abnormal microbiota that prevention is not achievable with short term dietary interventions aimed at

remodelling the gut microbiome. Whilst this association of diet and microbiome composition is increasingly well understood, what is less well understood is the effect on metabolite production by the gut bacteria in response to microbiome alterations (222, 223).

Despite the potential for huge variation in gut microbiome composition between individuals, studies have shown that some recurring bacterial species are regularly recovered from different individuals. The human gut microbiome is dominated in particular by 3 phyla: Firmicutes (30-50%), Bacteroidetes (20-40%) and Actinobacteria (1-10%). There is great variability within the GI tract. Bacteroidetes and Actinobacteria species comprise approximately 90% of colonic bacteria, but only 50% of small intestinal bacteria, where the predominant species are Firimicutes (214).

In health, the gut microbiome helps to form a natural barrier against pathogenic infections. This is aided by thick mucus layers which prevent excessive exposure of the enterocytes to micro-organisms and dietary antigens. Studies in germ-free mice, who share a very similar microbiome composition to humans; have shown that these animals have longer intestinal villi, crypt atrophy, reduced epithelial cell turnover and less angiogenesis (224).

The microbiome also plays a major role in maintaining normal gut homeostasis through, not only its protective function, but also its metabolic functioning. Studies in germ-free mice show that animals reared in bacteria-free conditions are

more prone to infections, have reduced digestive enzyme function, vascularity, their muscle wall thickness is reduced, along with a reduction in their serum immunoglobulins and cytokine production. They also have fewer intraepithelial lymphocytes and Peyer's patches throughout the gut (225). Restoring the gut microbiome in germ-free mice has led to improved mucosal immune systems, and increased expression of genes relating to nutrient uptake, metabolic process, angiogenesis and development of the intestinal nervous system (226). The gut microbiome has also been shown to influence immunity by facilitating development of humoral components of the mucosal immune system, and by affecting T-cell levels and T-helper cell cytokine release (214).

Finally, the gut microbiome has a role in the prevention of intestinal colonisation by pathogenic organisms. Whilst the underlying mechanisms for this function are unclear, it most likely represents a combination of competition for adhesion molecules and nutrients, the production of antimicrobial compounds, and stabilisation of the gut mucosal barrier (227, 228).

The fact that a low fibre, high animal fat diet results in an altered microbiome raises the possibility that the higher CRC risk seen in those eating a western diet, could be due to dietary effects on the microbiome, rather than the consumption (or not) of these food types e.g red meat/fibre, themselves.

Studies have been conducted into the microbiome composition of CRC patients and have demonstrated that the microbiome is perturbed and, indeed, that the

diversity is greatly reduced in CRC. This has been referred to as dysbiosis (214, 218). Specific bacteria identified as being over-represented in patients with CRC include; *Streptococcus bovis, Helicobacter pylori, Bacteroides fragilis, Enterococcus faecalis, clostridium septicum, Fusobacterium nucleatum, Providencia* and *Escherichia coli* strains (214, 221, 229). Conversely, butyrate-producing bacteria, such as *Roseburia* and *Fecalibacterium* are greatly reduced in CRC patients compared with controls (221, 230).

Whilst these different microbiome profiles have been observed, these findings do not establish whether these changes in microbiome composition are the cause of CRC, or a consequence of its development. A further problem with the studies described above, is the methodological variation between them. Some have utilised colonic tissue, whereas others have utilised faecal matter. A study aimed at addressing these shortfalls studied faecal and mucosal samples from both the tumour, and also sites proximal and distal to the tumour. The results showed that the microbiome of CRC subjects was different from the healthy controls, but that the alterations were not restricted to the cancerous tissue. Differences were detected throughout the whole colon. They also found a different profile for right and left sided tumours and that faecal microbiota was only partially reflective of the mucosal microbiota profile. Interestingly, they still observed great variation within the CRC subjects, and that they could be stratified into four groups based on the relative abundance of various bacterial species, raising the possibility of disease "enterotypes" (231). This is supported by previous studies which have

suggested the use of a microbiome profile, rather than a single bacterium, as a screening tool for CRC (232-234).

The mechanisms by which gut microbiome could precipitate or protect against CRC are multiple:

- Dietary and digestive components undergo metabolic processing by the microbiome with resulting products potentially acting as putative oncometabolites, including reactive oxygen species and tumour suppressive metabolites. Examples of this process include the processing of red meat and bile acids from animal fat to hydrogen sulphide and secondary bile acids, respectively. Elevated secondary bile acids have been found in CRC patients relative to healthy controls (229). Protective metabolites include equol, urolithins and short chain fatty acids (SCFAs) such as butyrate, which are derived from the microbiome activity on plant-based polyphenols and dietary fibre (235).
- Microbiota can affect inflammatory processes within the gut via the production of toxins and virulence factors. Toxins from certain bacterial species, namely *F.nucleatum, H.pylori and B. fragilis*, have all been linked with CRC (214). Chronic gut inflammation is a potential factor in development of CRC, as seen in patients with long standing IBD (218). As discussed earlier, aspirin and NSAIDs have been demonstrated to reduce the risk of developing CRC (58, 59) and this would suggest that anti-

inflammatory effects have a protective mechanism against CRC development.

- The microbiome can also affect gut permeability, and influences the exposure of luminal bacteria and bacterial antigens, such as lipopolysaccharides and flagellin, to the hosts immune cells and enterocytes, potentially leading to further inflammation (218).

Several theories of how the above changes can lead to the development of CRC have been proposed. The "alpha bug theory" proposed by Sears et al, in 2011, suggests that there are certain predominant bacterial species, which due to their metabolic processes or effects on the gut epithelium, lead to carcinogenesis. They may also have a role in terms of "crowding out" potentially carcino-protective bacteria (229, 236).

Another proposed theory is that of the "driver-passenger" model. This suggests that certain bacteria, the drivers, may produce the initial damage to the intestinal epithelium which can lead to CRC development. The progression of this initial damage to CRC, is promoted by the so called "passengers", which are opportunistic pathogens able to proliferate in the altered microenvironment caused by carcinogenesis (237).

A final suggested model is the "intestinal microbiota adaptions" model, which suggests that CRC and dysbiosis may have a symbiotic relationship. The CRC environment is characterised by host-derived immune and inflammatory

processes that would affect microbial regulation. This could, potentially, alter microbiome composition and favour the proliferation of pro-carcinogenic bacteria, thus amplifying the effect of dysbiosis, and further promoting CRC progression (238-240).

CHAPTER 3

Materials and Methods

3.1. Ethical Approval

Scientific and ethical approval was granted by the University Hospitals Coventry and Warwickshire (UHCW) Research and Development Office, as well as Solihull Ethics committee, ref: 13/WM/0136. Written informed consent was obtained from all who participated in the study.

3.2. Subject recruitment and sample collection

3.2.1. Patient recruitment

Patients were recruited between September 2015 and December 2016 from the Lower GI MDT list at UHCW. Patients with confirmed CRC, who had been informed of the diagnosis and management plan, were approached. Information sheets and a cover letter were posted out to them, and a telephone call made several days after this to follow up. Patients who were willing to take part in the study were sent a specimen collection pack, inclusive of urine and stool collection bottles, a consent form and participant questionnaire. The patients produced the samples at home on the same morning as they were due in for their operation, or first session of chemotherapy. Patients who were not receiving active treatment returned the samples to the pathology department at UHCW either in person, or, via a same morning courier service utilised by General Practitioners (GPs) in the area. A subgroup of patients with historical CRC who had previously undergone surgical resection within 1-3 years of the study start date, had previously given

urine samples for the FAMISHED study at UHCW. These patients were re-approached to take part in this study. They were recruited in the same way.

Inclusion criteria were adult patients with confirmed CRC, that was not due to a hereditary condition, and relatives and spouses/co-habitors who had consented. Exclusion criteria were patients who had concurrent malignancy, gastrointestinal conditions such as bile acid diarrhoea, coeliac disease and inflammatory bowel disease, or urological/renal conditions requiring secondary hospital care.

3.2.2. Relative and co-habitor recruitment

The initial cover letter sent to out to patients, informed them that we would only approach relatives and spouses/co-habitors who had given their consent to be contacted. Once this consent was passed on by the CRC patients, information sheets and cover letters were posted to the relatives and spouses/co-habitors. They were then contacted by telephone several days later to follow up. Those willing to participate were sent a specimen collection pack in the post, as for the patients. These were returned either by the GP courier service already mentioned, returned directly to the hospital on visiting their spouse/relative, or, for those more distant by post in appropriately marked and sealed packages.

3.2.3. Urine collection, storage and transfer

Urine samples were collected from patients directly, or via GP courier, and stored at -80^{o}C within two hours of receipt of samples. They were transferred to Owlstone, Cambridge in a box of dry ice and stored at –20^{o}C. They were then

defrosted in a laboratory fridge at 3^{o}C overnight, prior to analysis. Urine samples to be analysed at the University of Warwick were collected and stored in the same way, transferred to the University in dry ice and stored in a –20^{o}C freezer, and then defrosted in a laboratory fridge at 3^{o}C overnight, prior to analysis.

3.2.4. Faeces collection, storage and transfer

Faeces samples were collected from patients directly, or via GP courier, and stored at -80^{o}C within two hours of collection. They were transferred to the University of Warwick in dry ice and stored in a –80^{o}C freezer, and then defrosted for 45 minutes in room air, prior to analysis.

3.3. Sample analysis

3.3.1.1 LC-ultraFAIMS-MS

Initially the intention was to utilise the Lonestar FAIMS machine at the University of Warwick Engineering department. However, this device was experiencing recurrent faults, and break downs. This resulted in a four month delay between January and April 2016, waiting for the machine to be repaired. There was a subsequent delay due to commercial work on the machine which the engineering department had taken on. There were then configuration issues with a custom built autosampler in June – July 2016. As a result of this, the decision was made with my supervisors to approach Owlstone, the company which made the Lonestar FAIMS machine, directly. An agreement was reached to allow me to analyse the urine samples myself using some of Owlstone's equipment in Cambridge.

The equipment used was a hybrid machine, an LC-FAIMS-MS, which was based on the previously utilized FAIMS technology but had an Agilent 1200 series Liquid Chromatography (LC) column in series with the FAIMS, which allowed greater separation of VOCs prior to their passing through the FAIMS. There was also an autosampler (chilled to 4^{o}C) and an Agilent 6230 Time of Flight Mass Spectrometer incorporated into the set-up.

This methodology refers to the urine sample analysis conducted in Chapters 5 and 6.

The samples were stored at –20^{o}C and defrosted in batches of 25 randomly selected samples in a laboratory fridge at 3^{o}C overnight, prior to analysis.

Sample preparation was done as per Owlstone standard operating procedures:

1. LC column conditioned using a 20ml pooled batch, consisting of 2ml samples from 10 randomly selected urine samples (CRCs and relatives/co-habitors). This also served for quality control runs

2. 500µL of pooled urine was mixed in an LC autosampler vial with 500µL of 5% acetonitrile:95% LCMS grade water + 0.1% formic acid added to produce a 1:1 dilution

3. 10µL of pooled diluted urine applied to LC column (Agilent Poroshell 120 EC-C18 3.0 x 50mm, 2.7um with a guard column).

4. The flow rate on the column was 0.85ml/minute and the column temperature was maintained at 30^{o}C

5. The chromatographic gradient used was as below:

Time (mins)	% Mobile Phase A	% Mobile Phase B
0	95	5
0.01	95	5
1.0	95	5
7.0	60	40
7.5	10	90
8.5	10	90
9.0	95	5
11.0	95	5

Mobile Phase A: LC-MS grade water with 0.1% formic acid

Mobile Phase B: Acetonitrile with 0.1% formic acid

6. The urinary VOCs are separated based on their affinity for the LC column and were passed through an Electrospray to aerosolise the VOCs, which then passed through the FAIMS chip and on into the MS.

7. A blank consisting of 95:05 water:acetontrile with 1% formic acid was analysed in between each urine sample

8. 10 cycles of pooled urine were run in this fashion to condition the column

9. 1ml aliquots of thawed urine were placed in Eppendorf micro-centrifuge tubes and centrifuged at 10,000rcf for 10 minutes

10. 500µL of supernatant was mixed in an LC autosampler vial with 500µL of 5% acetonitrile:95% LC-MS grade water + 0.1% formic acid to produce a 1:1 dilution

11. 10µL of 1:1 sample dilution were applied to the LC column via the autosampler and analysed as above

12. A blank consisting of 95:05 water:acetontrile with 1% formic acid was analysed in between each urine sample

13. After every 5 urine samples a QC sample from the pooled urine was run as described

14. Before each run was started the next day, the Electrospray shield was cleaned to prevent urinary sediment build up

FAIMS parameters were as follows:

- Initial Compensation Field: –900
- Final Compensation Field: 4000
- Number of Compensation Field steps: 10
- Number of repeats: 590
- Initial Dispersion Field: 250000
- Final Dispersion Field: 250000

MS settings were as follows:

- m/z start: 80
- m/z end: 1700
- Scan rate: 12.0
- Gas temperature: 150^{o}C
- Gas flow: 10 l/min
- Nebuliser: 20psig
- Sheath Gas temperature: 250^{o}C
- Sheath Gas Flow: 12l/min
- VCap: 3500V
- Nozzle voltage: 2000V
- Fragmentor: 200V
- Skimmer 1: 65V
- Octopole RF peak: 750V

3.3.1.2 Statistical analysis

3.3.1.2.a Data processing

The LC-FAIMS-MS apparatus provided a four dimensional output of ion counts – one dimension for each of LC and MS, and two for FAIMS, however, as the dispersion field for the FAIMS was kept constant this resulted in a three dimensional output. Each LC-MS spectrum was run over 10 compensation field (CF) FAIMS settings. The two settings which provided the best yield in terms of extractable features were then selected for analysis.

As a result of the modified instrument set up used, the acquired LC-FAIMS-MS files were pseudo MS/MS files, with each collision energy setting corresponding to one of the 10 FAIMS CFs. All the available software for chromatographic feature extraction are designed to perform feature extraction on the total ion current (TIC) chromatogram. In this case, LC-FAIMS-MS, the TIC chromatogram varies significantly from standard LC-MS data. As a result, of this it was not possible to perform feature extraction directly on the raw LC-FAIMS-MS data files. To solve this, the acquired data was split into individual chromatograms corresponding to acquired FAIMS CF settings, (designated 05_100-57 and 06_101-06) and saved as individual files.

A custom python script written by Owlstone was used to split and extract the CFs as separate files, and simultaneously save them in the required format (.mzML). The saved .mzML files were subjected to feature extraction using XCMS package in R (https://bioconductor.org/packages/release/bioc/html/xcms.html). This is an

open source tool which can be used for feature extraction from chromatographic data. Feature extraction was then performed on each individual file, and the output .csv file created contained the list of features for the analysed sample. These output files were subsequently amalgamated into files based on the day on which the samples were analysed. This process is summarised in figure 3.1.

An example of the output .mzML file can be found in figure 3.2, whilst an example output .csv file containing the extracted output feature data can be seen in figure 3.3.

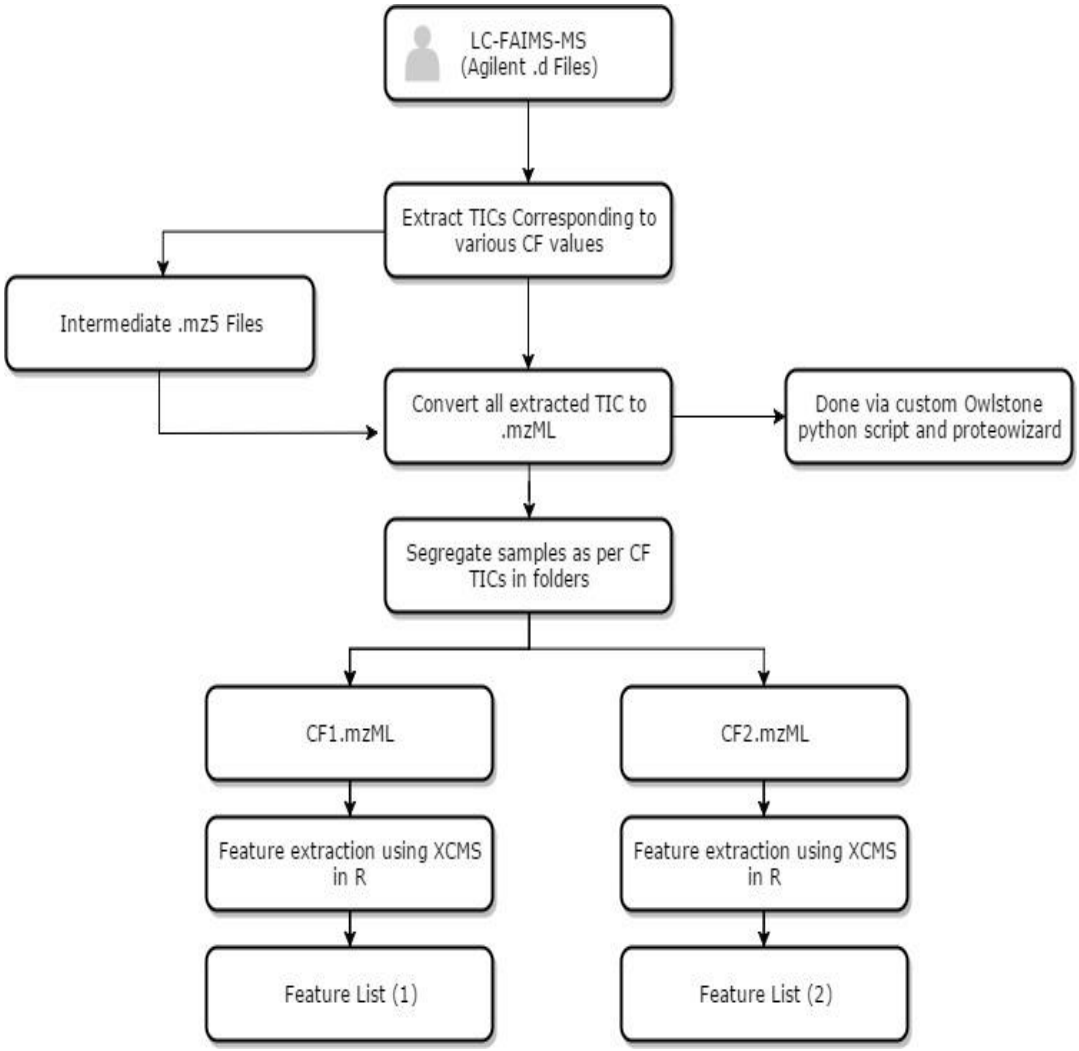


Figure 3.1. Workflow summary of data processing and extraction process.

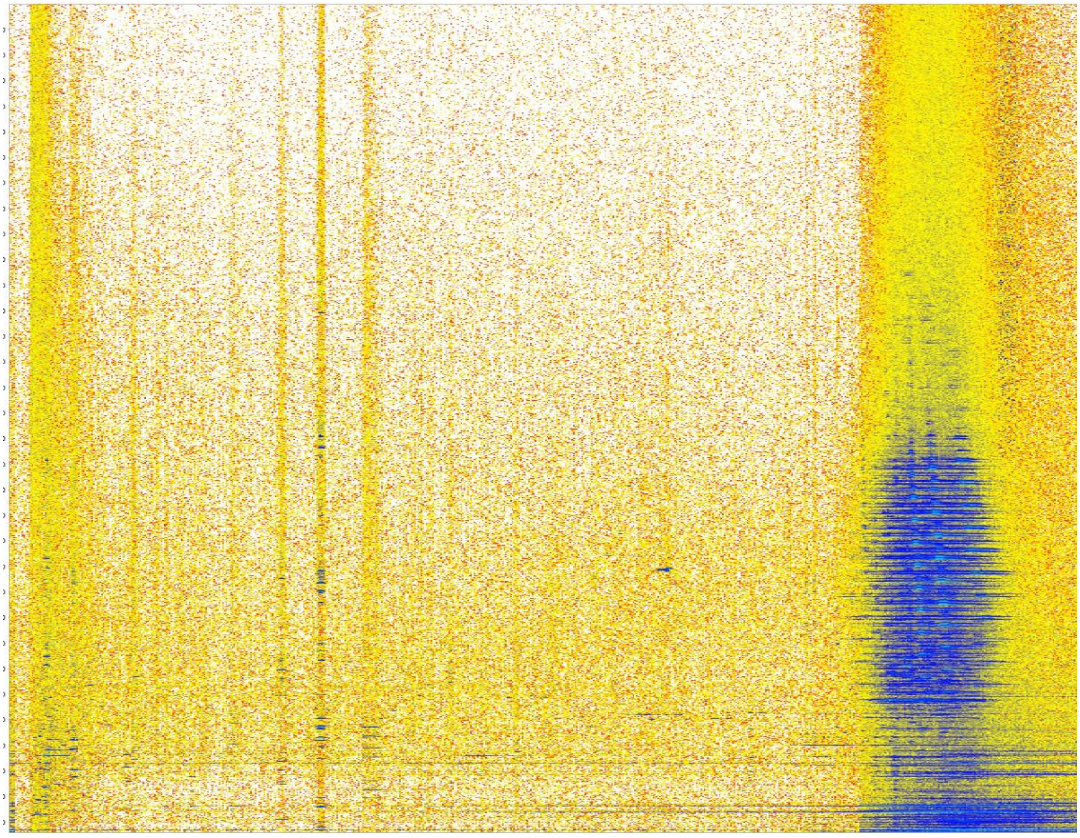


Figure 3.2. Example .mzML output file displayed on a log colour map using Zmine.

mzmed	mzmin	Mzmax	rtmed	rtmin	rtmax	npeaks	maxint	CRCN-001pre	CRCN-023pre
80.0615	79.997	80.0948	9.6419952	9.3582483	9.858802	166	9770	26876.90675	499850.2631
80.0487	80.038	80.0692	0.0657063	0.0307366	0.124988	65	335	395.2577235	460.4560128
80.0589	80.033	80.0845	10.571786	10.277567	10.9782	77	2141	1321.677	24091.83467
80.0589	80.033	80.0948	9.1469969	9.1034764	9.206579	8	7494	66166.68187	51919.01005
80.1281	80.099	80.1973	9.6396184	9.3181235	9.877527	136	279	9086.728646	9377.65262
80.2358	80.217	80.2537	9.6958347	9.5981027	9.744488	4	115	489.3336	853.02888
81.0302	81.019	81.0406	2.8058001	2.7881938	2.840769	25	233	94.81971163	868.3385571
81.0302	81.019	81.0406	1.0641199	1.0183309	1.124352	9	718	310.2324673	221.3541752
81.0406	81.040	81.0457	9.407566	9.2034002	9.421078	5	664	9415.946081	164.2185484
81.0509	81.030	81.087	9.6773322	9.5209395	9.870154	55	852	14569.77685	3345.788571
81.0715	81.056	81.0973	4.0271378	4.0157358	4.157116	32	1083	229.8967707	235.7741977
81.0457	81.040	81.056	0.3898414	0.3600242	0.407732	11	328	470.353397	652.0176
81.0612	81.050	81.0921	9.3792202	8.8379852	9.465016	43	847	20676.451	2700.774574

Figure 3.3. Example section of output .csv file containing the extracted feature data. Each file contained 5000+ data points per sample.

3.3.1.2.b. Statistical methods

As this technology is novel, there is very limited statistical literature available on an optimal analysis technique. The assistance of a bioinformatician from the University of Warwick was utilised to develop a potential pipeline for analysis. The bioinformatician has previously worked on other VOC projects from our research group. It was felt he was best placed to develop a usable and robust analysis method.

Prior to analysis, data from the LC-FAIMS-MS was processed via a bespoke software pipeline, which was developed specifically for this project. Individual data files were read in and linked to their clinical grouping. The data for each sample consisted of a set of features, or peaks, which were extracted from the raw data as described in section 3.3.1.2.a. These peaks were aligned and grouped together on a standardised grid, based of mass-to-charge-ratio and retention-time values. This forced alignment allowed direct comparison across samples, and also improved the signal-to-noise of the data, due to the presence of large numbers of isolated, likely spurious, peaks in the raw data files.

Once aligned, these peaks formed a set of standard features for each sample, which was then suitable for analysis. Any features which were found to have zero variance across samples were removed automatically, as they would be uninformative. Any sample for which there were no data (i.e. data outputs all zeros), was also similarly removed, as this would represent an analysis failure by the LC-FAIMS-MS machine. Any feature containing data for <10% of the samples

was also removed, as it would be too sparse to be useful. The pre-processing of the data was completed by normalising the remaining features so that they had zero mean and unit variance.

For each separate analysis of subgroups of the data, the relevant subset of samples was extracted, and the different groups defined. 5-fold cross-validation was then used to assess classification accuracy across these groups, using three different multi-class classifiers: sparse logistic regression, Support Vector Machine, Random Forest. This analysis generated outputs of one-vs-all Receiver Operator Curves (ROC) i.e. comparing a single group vs all other groups, for example CRC vs relatives and spouses, relatives vs CRC and spouses, spouses vs CRC and relatives. Other results generated included the Area-Under-Curve (AUC) statistic, sensitivity/specificity values, which were selected automatically to be maximally similar given the ROC curve, and a p-value, comparing the result to that expected for random chance (AUC=0.5), using a Wilcoxon rank-sum test.

All analyses were carried out using the R programming language (v3.3.1).

3.3.2. FAIMS

During the planning of the main thesis experiment, a potential issue was identified with the storage of urine samples prior to being placed in the freezer. Given the fact that some samples were being collected from relatives who live distant to the CRC patients, this necessitated the use of the postal service for return of samples. This means that potentially, although first class postage was used, samples produced for example on a Monday morning and posted that same day, may not reach the freezer at UHCW until Wednesday, although, they were transferred in sealed universal bottles. This means that the urine would be at room/atmospheric temperature for 48 hours and, potentially, up to 72 hours. It has been demonstrated that the VOC profile of blood samples can be affected by different storage conditions (241), but no studies have been conducted on urine samples.

A further experiment was conducted whereby urine samples were collected from 27 healthy volunteers, and informed consent was taken. Aliquots of urine were frozen immediately, and then further aliquots were left in sealed universal bottles at room temperature for increasing time periods of 12 hours, 24 hours, 36 hours, 48 hours and 72 hours, before freezing at -80^{o}C. The urine was then transferred to the Engineering department at the University of Warwick, in dry ice, for analysis. The aim of this experiment being to determine the effect of prolonged storage at atmospheric temperature of urine samples.

A commercial FAIMS instrument was utilised, specifically the Lonestar device (Owlstone, UK). This device is separate from that described in section 3.3.1 and is

the machine which was originally planned to be used for all urinary sample analysis in this thesis.

FAIMS achieves separation of complex chemical mixtures as a result of the different molecular mobilities of the chemicals as they are subjected to high and low electric fields as the chemical mixture passes through the analysis chamber. This allows separation of gas molecules at atmospheric pressure and room temperature. This, in turn, allows identification of the chemical composition of VOCs, and detection of volatile molecules present at very low concentrations. An overview of the FAIMS process can be found in Figure 3.4.

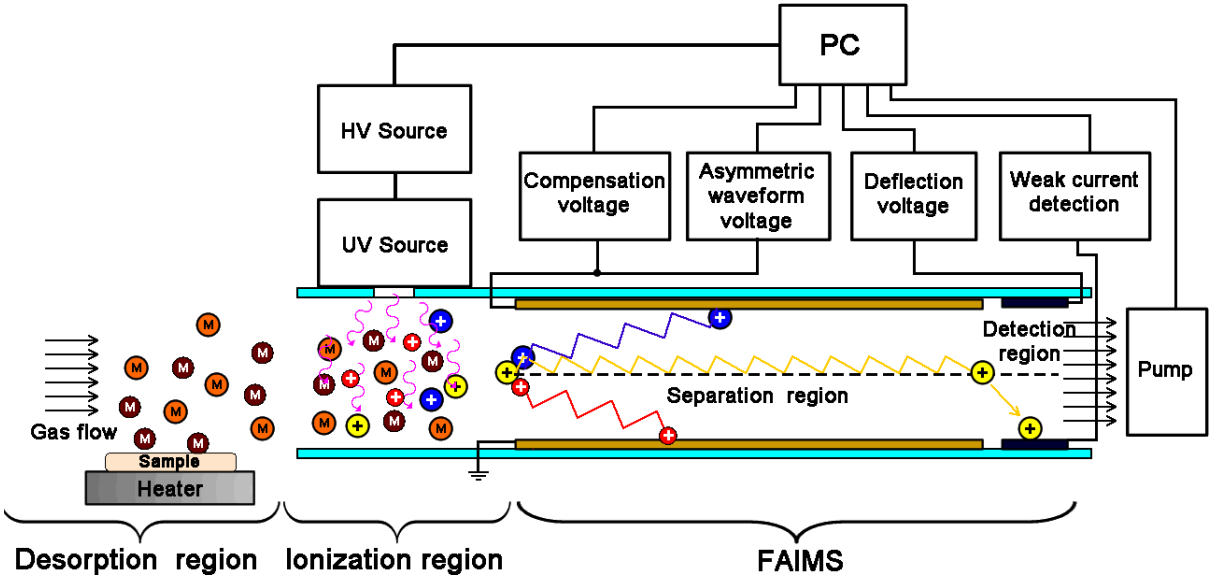


Figure 3.4. Diagram of the FAIMS process (adated from Owlstone Standard Operating Procedures). The sample is heated and moved through the ionization phase by gas flow. The ionized molecules are then subjected to high and low voltage electric fields. This results in separation of the molecules based on their mobility in response to the fields.

The FAIMS analysis was conducted by myself at the University of Warwick Engineering department as per the Engineering Departments standard operating procedures.

This methodology refers to the urine samples analysed in Chapter 4.

5mls of each individual urine sample were aliquoted into a 22ml borosilicate glass vial (Fisher Scientific, UK) with a rubber bunged cap, and placed in a custom built chilled auto-sampler tray at 4^{o}C, before sampling via an ATLAS sample system (Owlstone UK) attached to the Lonestar FAIMS machine. The ATLAS system heats the sample to 40±0.1^{o}C and it is then sampled. The dispersion field was stepped through 51 equal settings ranging from 0% to 90% of the magnitude of the electrical field. For each dispersion field setting, the compensation voltage was stepped between +6V and –6V in 512 steps.

Each sample aliquot had fourteen analyses performed sequentially with a blank run performed between each analysis. Sample analysis was run over 90 - 120 seconds, with equivalent times for blank runs. Each sample run has a flow rate over the sample of 2 l/min of clean, dry air. After each sample, clean dry air is run through the sensor ten times sequentially as a blank, to "zero" the sensor. Batches of 20 samples were loaded onto the chilled autosampler for analysis.

3.3.2.1. Statistical Analysis

Each subjects urine aliquot underwent fourteen sequential samplings for analysis on the FAIMS. Each sample produced output data described hereafter as a matrix. This matrix represents a plot of signal intensity for the respective dispersion fields and compensation voltages. As there are both positive and negative ions being detected, this results in 14 positive and 14 negative FAIMS matrices for each time point for each subject. The separation of these ions through the high and low electric fields is a result of their mass to charge ratio and results in the characteristics FAIMS "plumes", which are graphical representations of the matrices. Figure 3.5a and 3.5b give examples of positive and negative FAIMS plumes, which correspond to an individual matrix.

The output data has a very large number of datapoints, 52,224, accounted for by 51 dispersion field settings, each with 512 compensation voltages and both positive and negative matrices. When looking for disease states these datasets need to be concatenated into a 1D array, and then a Daubechies D4 wavelet transformation used to extract important features. This allows reclassification, whilst preventing minimal important signal loss. Once this has been completed any co-efficients which fall below a given threshold would be excluded, as these are predominantly background noise.

However, in this case, the concatenation and transformation were not performed. This is because the samples were not going to be reclassified and, therefore, all data was included in the analysis, including low intensity signals.

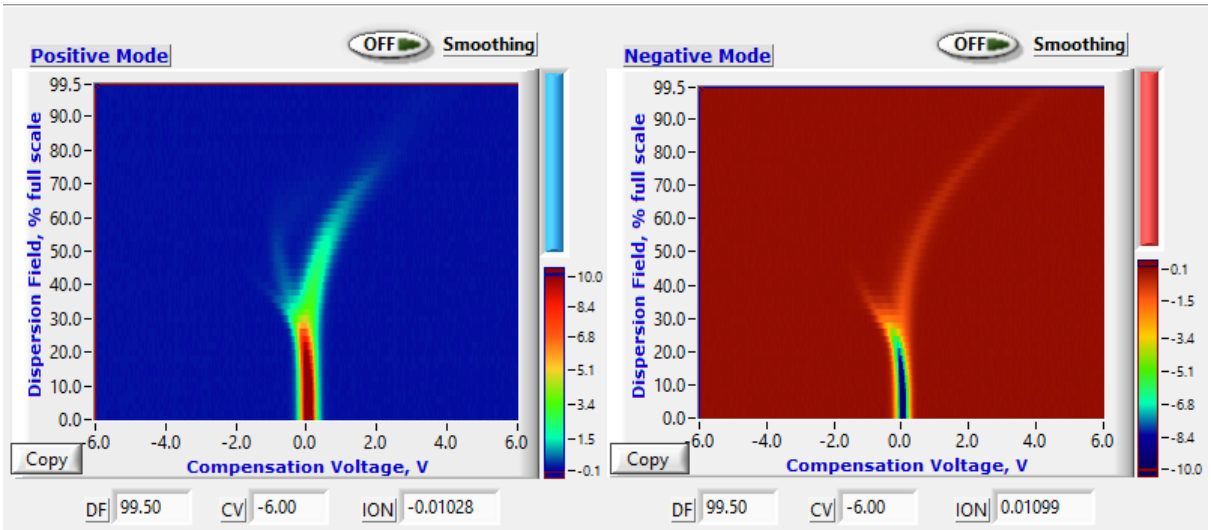


Figure 3.5a. Example of a positive FAIMS plume, which is a graphical representation of the positive ions from the FAIMS matrix for a given sample

Figure 3.5b. Example of negative FAIMS plume, which is a graphical representation of the negative ions from the FAIMS matrix for a given sample

3.3.2.2. Variation of mean of matrices from baseline:

To analyse the variation of the urinary VOCs from each healthy subject, at each time point, the arithmetic mean of all 14 positive and 14 negative matrices from time point zero, were compared to the arithmetic mean of all matrices from that patient, at each subsequent time point.

Notation used:

Matrix: Mp,t,n,c,s

p: Patient number

t: time (0,12,24,36,48 or 72 hours)

n: spectrum number at each time point (1-14)

c: compensation voltage index

s: Separation field index

P: total number of patients: 20

T: number of time points: 6

N: number of spectra for each sample: 14

V: variation

C: total ion count

The mathematical formula used was:

$$\text{Variation}(t) = \Sigma p\, Vp,t$$

Where: $$Vp,t = \frac{\Sigma c \Sigma s \left| \Sigma n Mp,t,n,c,s - \Sigma n Mp,t=0,nc,s \right|}{\Sigma c \Sigma s \left| \Sigma n Mp,t=o,n,c,s \right|}$$

Spearman's rank correlation coefficient was used to test for a monotonic relationship between time and variation of the mean of the matrices from baseline.

3.3.2.3. Variation of total ion count from baseline:

The relative variation in the total ion count in the FAIMS matrices was also calculated as a function of time.

The arithmetic mean of the number of ions detected for each patient at each time point was calculated. This data was then compared to the arithmetic mean of the ion count for that patient, at time zero.

The mathematical formula used was:

$$\text{Count}(t) = \frac{\Sigma p\ Cp,t}{P}$$

Where: $$Cp,t = \frac{\Sigma n \Sigma c \Sigma s |Mp,t,n,c,s|}{\Sigma n \Sigma c \Sigma s |Mp,t=o,n,c,s|}$$

Spearman's rank correlation coefficient was used to test for a monotonic relationship between time and variation of the mean total ion count from baseline.

3.3.3. 16s rRNA sequencing

Analysis of the stool samples was carried out by myself at the University of Warwick Life Sciences department as per the following methods.

3.3.3.1. Bacterial DNA extraction

Stool samples were defrosted at room temperature for 45 minutes. DNA extraction was performed using QIAamp Fast DNA stool mini kits (QIAGEN). 200mg of stool weighed and placed in a QIAamp Mini spin column. 1.4ml of ASL buffer was added to the spin column to lyse human and bacterial cells present within the stool. The columns were then pulsed for 2 x 40 seconds in a Fast-Prep 24 5G lysis machine (MP Bio). The lysed samples were then incubated at 95°C for 10minutes, after which they were briefly vortexed to ensure the stool was fully homogenised. After this, the samples were then centrifuged at 1,200rpm for 4 minutes to precipitate the stool residue. The supernatant was then removed and placed in a fresh 2ml micro-centrifuge tube. 1ml of InhibitEX buffer was added to the tubes to bind potential PCR inhibitors from the lysed cells. Following this, the samples were vortexed for 1 minute to ensure complete homogenisation. 1.2ml of the InhibitEX:supernatant mixture was transferred to a fresh micro-centrifuge tube and the mixture was incubated at 95°C for 30 minutes. The samples were then vortexed for 1 minute to ensure complete homogenisation, followed by centrifugation at 17,000xg for 1 minute to precipitate out the residue.

200µl of supernatant was transferred to a fresh 2ml micro-centrifuge tube containing 15µl of Proteinase K. 200µl of buffer AL was then added to the micro-centrifuge tube, which were vortexed for 15 seconds and subsequently incubated at 70°C for 10 minutes. The Proteinase K degrades and digests proteins within the solution, and AL is a lysis buffer. The tubes were then briefly centrifuged at 17,000xg to remove any drops from the lids of the micro-centrifuge tubes and 200µl of absolute ethanol (96-100%) was added to form a lysate. After this, the samples were vortexed for 15 seconds to mix thoroughly and briefly centrifuged again at 17,000xg.

600µl of lysate was applied to QIAamp spin columns and centrifuged at 17,000xg for 1 minute. The collection tube and its filtrate were discarded and the column was placed in a new collection tube. 500µl of buffer AW1 was applied to the column and centrifuged at 17,000xg for 1 minute. The filtrate was again discarded, and the column placed in a new collection tube, after which 500µl of buffer AW2 was added to the column. This was then centrifuged at 17,000xg for 3 minutes 30 seconds. The filtrate and collection tube were again discarded, the column placed in a fresh tube, and re-centrifuged at 17,000xg for a further 3 minutes 30 seconds. AW1 buffer contains a higher proportion of ethanol, which removes excess salt and improves pH conditions within the column. AW2 buffer undergoes a longer centrifugation to remove impurities and digested proteins. The extracted DNA is removed from the column by applying 100µl of ATE buffer, incubating at room temperature for 5 minutes, and then centrifuging at 17,000xg for 1 minute.

3.3.3.2. DNA quantification

The concentrations of extracted DNA solutions were checked using Qubit 2.0. Stock solutions were made of 1:200 dilutions of Qubit dsDNA reagent (Life technologies, USA) in dsDNA buffer. 0.5ml tubes were used for quantification. 190µl of stock solution was added for standards, and 199µl for samples. 10µl of Qubit standards were added to the standard tubes and 1µl of samples was added to the sample tubes. The samples were mixed by vortexing, and the DNA concentrations checked on the Qubit fluorimeter against the standards. The concentrations were recorded.

3.3.3.3. Polymerase Chain Reaction

The V3-V4 fragment of the bacterial 16s region from the extracted DNA was amplified by polymerase chain reaction (PCR). The primers used are listed below:

V3-V4 Forward primer	5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG 3'
V3-V4 Reverse primer	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGT ATCTAATCC 3'

2.5µl of 5ng/µl extracted DNA was placed into PCR reaction tubes with 10µl of pooled forward and reverse primers and 12.5µl of PCR Mastermix (ThermoFisher), to make a total volume of 25µl.

This was then run through the following PCR programme:

Temperature and Time	Number of Cycles	Purpose
95**°C for 3 minutes**	1	Initial Denaturation
95**°C for 30 seconds** 55**°C for 30 seconds** 68**°C for 30 seconds**	25	Denaturation Annealing Extension
68°C for 5 minutes	1	Final Extension
12°C hold	1	Hold

The PCR products were then analysed by electrophoresis on a 1% agarose. 1g of agarose was dissolved in 1x Tris-acetate (TAE) buffer, and 10µl SYBR safe (Life Technologies) was added to give a final concentration of 1:10,000 to allow DNA visualisation. The 1x TAE had been diluted with de-ionised water from a 50x stock solution. The stock solution consisted of 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5EDTA (pH8.0) for 1 litre.

The agarose was then melted in a microwave and the molten agarose poured into a plastic cast, with a plastic comb inset to produce the requisite wells. The comb was removed once the gel had set. The gel was placed in a horizontal

electrophoresis tank, filled with enough 1x TAE buffer to cover the gel. 5µl of PCR product was mixed with 5µl of Fermentas 6x loading dye (#R0611) and 5µl of the mix was applied to the gel. Hyperladder 2kb (Bioline, UK) was used as a size ladder for all gels. The gels were run at 100millivolts for 30 minutes, and the DNA visualised by transferring the gel to a BioRad gel-doc system. The size of the V3-V4 region is ~460 base pairs (bp) (see figure 3.6).

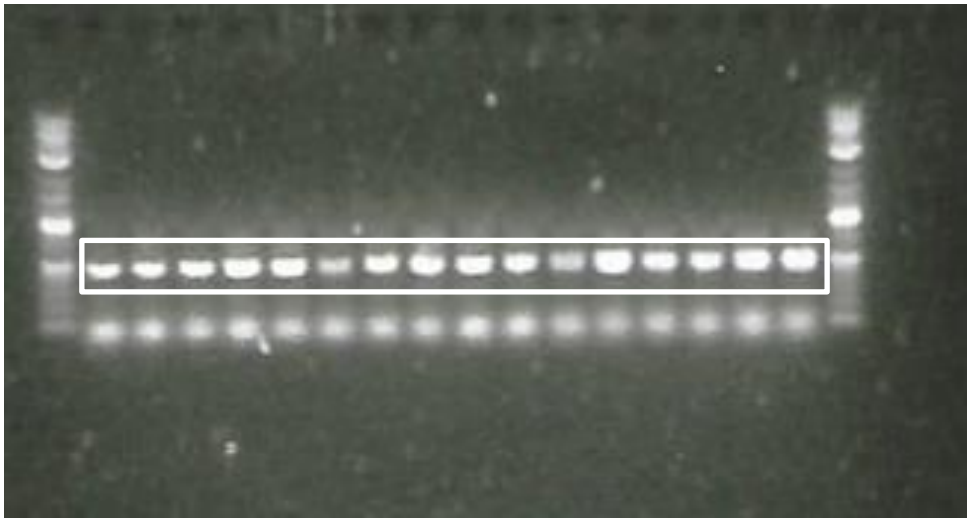


Figure 3.6. Gel electrophoresis of the initial PCR reaction from extraction bacterial DNA/RNA with 16S v3/V4 specific primers. First and last columns are 2kB Hyperladders. The V3-V4 region is ~460bp long, corresponding to the uppermost signal in each well – marked with a white box.

3.3.3.4. Post-PCR DNA purification

The remaining PCR products were transferred to fresh 1.5ml micro-centrifuge tubes. To each sample 20µl of AMPure magnetic beads were added, mixed by up and down pipetting, and then incubated at room temperature for 5 minutes, to allow the DNA to bind to the magnetic beads. The tubes were then placed in a Dyna-Mag2 magnetic stand (ThermoFisher) and left to stand for 2 minutes, to allow the beads to pellet against the magnet. The supernatant was then removed and discarded. After this, the beads were then washed twice with 200µl of absolute ethanol (96-100%), with the supernatant removed and discarded after each wash. The beads were left at room temperature to dry for 10 minutes after the second ethanol wash. They were then removed from the stand and mixed with 52.5µl of Elution Buffer (10 mM Tris-Cl, pH 8.5) (QIAGEN), and pipetted up and down 10 times to mix and elute the DNA from the AMPure beads. Following this, the beads were incubated at room temperature for 2 minutes, before returning to the magnetic stand for a further 2 minutes. After the magnetic beads had been pelleted, the supernatant, containing the DNA, was carefully removed and stored frozen at –20°C prior to the next PCR step.

3.3.3.5. Illumina 16s rRNA gene sequencing library preparation

A further PCR was performed using the cleaned DNA from the first PCR. The forward and reverse primers used in this PCR contained a unique barcode, to allow identification of 96 individual samples after sequencing. The table (table 3.1) overleaf shows how unique pairs of forward and reverse primers allowed for identification of each sample.

Primers	N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
S501	1	9	17	25	33	41	49	57	65	73	81	89
S502	2	10	18	26	34	42	50	58	66	74	82	90
S503	3	11	19	27	35	43	51	59	67	75	83	91
S504	4	12	20	28	36	44	52	60	68	76	84	92
S505	5	13	21	29	37	45	53	61	69	77	85	93
S506	6	14	22	30	38	46	54	62	70	78	86	94
S507	7	15	23	31	39	47	55	63	71	79	87	95
S508	8	16	24	32	40	48	56	64	72	80	88	96

Table 3.1. Table of unique forward and reverse PCR primers to allow identification of unique samples in the Illumina Miseq

The PCR was performed using **5**µl of purified DNA from the last step, 5µl of forward primer, 5µl of reverse primer, 10µl of molecular biology grade water and 25µl of PCR extensor mastermix.

The PCR was run using the programme below:

Temperature and Time	Number of Cycles	Purpose
95**°C for 3 minutes**	1	Initial Denaturation
95**°C for 30 seconds** 55**°C for 30 seconds** 68**°C for 30 seconds**	8	Denaturation Annealing Extension
68°C for 5 minutes	1	Final Extension
12°C hold	1	Hold

The PCR products were cleaned using AMPure magnetic beads as described above with the following alterations.

- 56µl of AMPure beads were used
- 27.5µl of Elution Buffer (10mM Tris pH8.5) was used.

The concentration of the purified DNA was checked using a Qubit 2.0, as outlined in section 3.3.3.2.

The samples were then diluted to 4nM concentration in 10mM Tris pH 8.5, and 5µl of the diluted DNA from each sample was added to a fresh 1.5ml micro-centrifuge tube, to form a pooled 4nM DNA library.

3.3.3.6. Illumina 16s rRNA sequencing

To denature the pooled DNA, 5µl of the pooled library was mixed with 5µl of 0.2N NaOH, vortexed, then centrifuged at 280xg for 1 minute, and left to incubate at room temperature for 5 minutes. This 10µl was then added to 990µl of chilled HT1 buffer and placed on ice. The pool was then diluted to a final concentration of 12pM by mixing 360µl of the pooled library with 236µl of chilled HT1 buffer and 6µl of 10nM PhiX control library. PhiX is a small, well-characterized genome, and it provides a quality control for sequencing and bioinformatic analysis. The final denatured DNA pool was subsequently sequenced on an Illumina MiSeq, using a MiSeq V3 2 x 300bp paired end protocol.

3.3.3.7 Processing of the 16S rRNA gene-fragment sequences

Sequenced files (.fastq) were de-multiplexed using default Illumina software. This process included the trimming of barcodes and primer sequences and also the removal of low quality reads. Forward and reverse reads were merged, using the UPARSE pipeline, to produce contiguous sequences. A quality filtering command was used to discard reads where there were three or more mismatches in the sequences. Where there were less than 3 mismatches, the base with the highest

quality value was inserted into the contiguous sequence. The merging and filtering steps were performed using the command:

usearch –fastq_mergepairs read1.fastq – reverse read2.fastq –fastq_maxdiffs 3 –fastqout output.fastq.

The merged and filtered sequences were then de-replicated and sorted by size and frequency, with singleton sequences excluded. This was done using the commands:

usearch_64_8 –derep_fulllength filter.fa –fastaout rep.fa –sizeout

usearch_64_8 –sortbysize rep.fa –fastaout rep_sorted.fa –minsize 2

Each contiguous sequence was subsequently assigned to an Operational Taxonomic Unit (OTU). An OTU is a unique bacterial sequence, which will encompass all similar sequences from the sequencing step.

The OTUs were then clustered and filtered to a default 97% identify level, and chimeras were removed. This was using the commands:

usearch_64_8 –cluster_otus re_sorted.fa –otus otus.fa

usearch_64_8 –usearch_global filter.fa –db otus_name.fa –strand plus –id 0.97 –uc readmap.uc

The OTUs were next assigned to each sample using the command:

assign_taxonomy.py –i otu_name.fa –m uclust –r /blastdb/gg_13_8_otus/rep_set/97.otus.fasta –t /blastdb/gg_13_8_otus/ taxonomy/97_otu_taxonomy.txt –o output_directory

After this, the 16s RNA OTU sequences were then assigned taxonomic classification using the command:

biom add-metadata –i otu_table.biom –o all.biom --observation-metadata-fp taxa.txt --observation-header OTUID,taxonomy,confidence

Once this processing was completed in UPARSE, then QIIME (Qualitative Insights Into Microbial Ecology) software was used to perform alpha diversity analysis using rarefied OTU tables. This was performed using the the command:

Alpha_rarefaction.py –I out_table.biom –o arare_max100/ -t rep_set.tre –m fasting_map.txt –e 100

QIIME was then used to calculate beta diversity using weighted and unweighted unifraction analyses using the command:

beta_diversity.py -i otu_tables/ -m weighted_unifrac -o beta_div/ -t rep_set.tre

The depth of rarefaction was determined by the lowest number of sequences assigned to a sample within a group that was analysed.

3.4. Literature search

A search of Pubmed was conducted using the search terms "Colorectal cancer" or “colorectal carcinoma" or "bowel cancer" and "volatile organic compounds" or "volatile organic substances" and "biomarkers". Search results were title and abstract screened. Additional references were obtained from citations within returned search results.

CHAPTER 4

Characterisation of VOC degradation over time at room temperature for healthy individuals

4.1. Introduction

Prior to the collection of samples for the main experiment of this thesis an issue was identified that had important practical implications for sample collection and storage. That issue is that very little is known about how VOC profiles are affected by varying storage conditions e.g. refrigeration or freezing, and how they are affected by prolonged storage at room temperature prior to freezing.

To date, there has been only a few studies into the effects of storage on VOC profiles of bodily samples. The first was conducted on the blood of healthy volunteers and compared analysis of fresh blood samples with samples refrigerated or frozen (241). It found that VOC profiles became more complex over time, and that frozen samples had a VOC profile distinct from samples stored in refrigerators and at room temperature. The second studied the effects of various storage parameters on the faecal VOC profiles of infants using an E-nose. They found that faecal sample size, water content, duration of storage at room temperature, faecal sample temperature, number of freeze/thaw cycles, and sampling method, all affected the VOC profiles. They concluded that the methodology of sample, collection, storage and analysis needs to be standardised, to allow for faecal VOC analysis to be utilised in a clinical setting (242). Thus far, there have been no studies of the effects of storage on VOC profile in urine.

Given the diverse way in which samples were being returned for the main experiment, (hand delivered, courier returned or by postal service) it became apparent that this could have implications for data interpretation and validity. It

also highlights the lack of a defined optimum protocol for the collection and storage of samples used for VOC analysis.

The experiment described in this chapter attempted to address some of these issues and determine whether the results of the main experiment were indeed valid.

4.2. Methods

Sample collection and storage was performed as described in sections 3.2.3. Samples were collected from 27 healthy volunteers. They were immediately divided into 6 aliquots and sealed into universal storage bottles. Aliquots were then stored at room temperature before individual aliquots were frozen after 0, 12, 24, 36, 48 and 72 hour time periods of room termpature storage. Samples were thawed and analysed using the Lonestar FAIMS machine (Owlstone, UK) at the University of Warwick, as described in section 3.3.2.

Once sample analysis was performed, the FAIMS data was searched manually to identify sample data from blank control samples. The identified data are described as matrices, which are a graphical representation or plume of the individual FAIMS data collected from each sample aliquot analysis. Each sample for a single subject and time point underwent 14 sequential samplings for FAIMS analysis. The FAIMs process generates both positive and negative ions, resulting in 14 positive and negative matrices for each subject at each time point.

The data then underwent statistical analysis as described in sections 3.3.2.1 and 3.3.2.2.

4.3. Results

27 healthy subjects were recruited to provide urine samples. 1 did not return any samples, 2 subjects provided insufficient urine for six aliquots and 4 samples were incompletely analysed due to technical errors with the FAIMS machine. A per protocol analysis was performed on the cohort of 20 complete sample sets.

The mean age of subjects was 49.6 years (Standard Deviation (SD) 9.6 years), and there were 6 males (23%). The mean BMI was 28 (SD 5.8) and there were 2 smokers (7.7%). Average alcohol consumption was 5.3 units per week per subject (SD 5.1). No subjects had significant genitourinary or gastrointestinal disease. 3 subjects (11.5%) had asthma, 2 hypothyroidism (7.7%) and 4 hypertension (15.4%). No subjects were taking NSAIDs, antibiotics or PPIs at the time of sample collection. All urine samples were collected in the morning prior to 9am.

4.3.1. Mean VOC Variation from Baseline:

For each patient, the arithmetic mean of all matrices from each successive time point (12, 24, 36, 48, 72), was compared to the mean of the same patients' matrices at time point zero, as described in section 3.3.2.2. This was done for both positive and negative FAIMS matrices. The relative variation of VOC signatures in the FAIMS matrices increased with time, from time point zero to 72 hours, for both positive and negative matrices. There was a plateau phase in the relative variation between time point 12 hours and time point 48 hours. This pattern was observed across all subjects for positive and negative matrices. Figure 4.1a shows the relative variation in the mean of the positive FAIMS matrices compared to time. Figure 4.1b shows the relative variation in the mean of the negative FAIMS matrices compared to time.

Using Spearman's rank correlation coefficient to test for a monotonic relationship between mean variation from baseline and time, gave $\rho 0.94$, $p = 0.017$ for positive matrices, and $\rho 0.83$ $p = 0.058$ for negative matrices. When all subjects had positive and negative matrix data pooled for each time point, Spearman's rank correlation gave $\rho 0.52$, $p < 0.001$ for positive matrices and $\rho 0.54$, $p < 0.001$ for negative matrices.

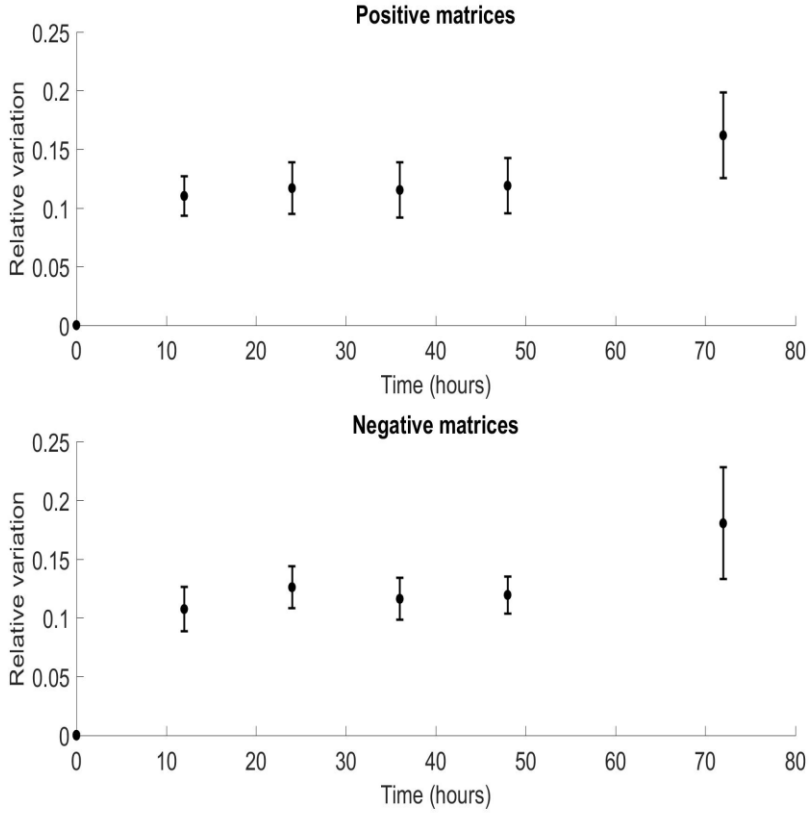


Figure 4.1a. Variation of positive FAIMS matrices from the mean of the matrices from t=0. Bars show standard error. Correlation of mean variation with time: Spearman ρ0.94, p = 0.017. Correlation of all points with time ρ0.52, p < 0.001. Plateau phase seen between 12 and 48 hours.

Figure 4.1b. Variation of negative FAIMS matrices from the mean of the matrices from t=0. Bars show standard error. Correlation of mean variation with time: Spearman ρ0.83, p = 0.058. Correlation of all points with time ρ0.54, p < 0.001. Plateau phase seen between 12 and 48 hours.

The variation from baseline was also reflected in the FAIMS spectral structures when averaged over all patients. Figures 4.2a and 4.2b show the baseline FAIMS plume structure, alongside the plumes for time points 12, 24, 36 and 72 hours, for positive and negative matrices respectively. They also show the difference in matrices between time point 0 and 72 hours for both the positive and negative matrices. In both cases there is a predominantly positive difference in the FAIMS spectrum which increases consistently with time, as shown by the FAIMS spectra from the other time points. This indicates an increase in the number of VOCs with increasing time.

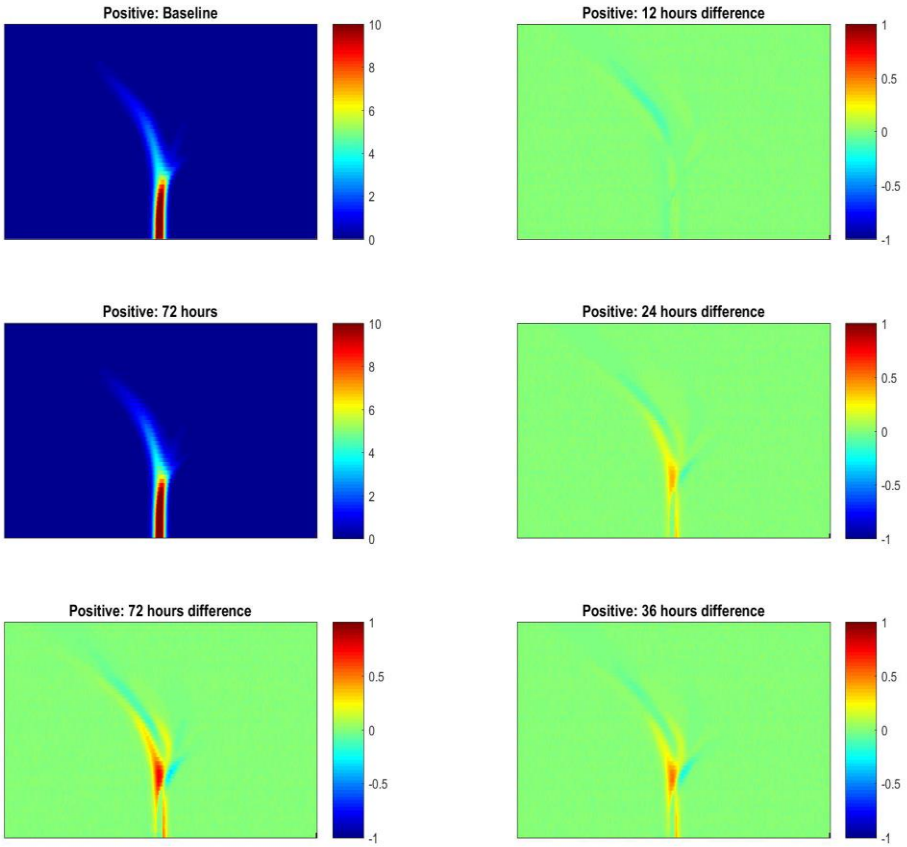


Figure 4.2a. Positive matrices, difference from baseline with time. Top left: FAIMS spectrum at time zero. Middle left: FAIMS spectrum at 72 hours. Bottom left: Difference between spectrum at 72 hours and baseline. This shows a predominantly positive difference spectrum indicating an increase in VOCs. Right hand panels show the difference at 12, 24 and 36 hours from time 0, showing a consistent pattern, developing with time. All matrices averaged over all patients.

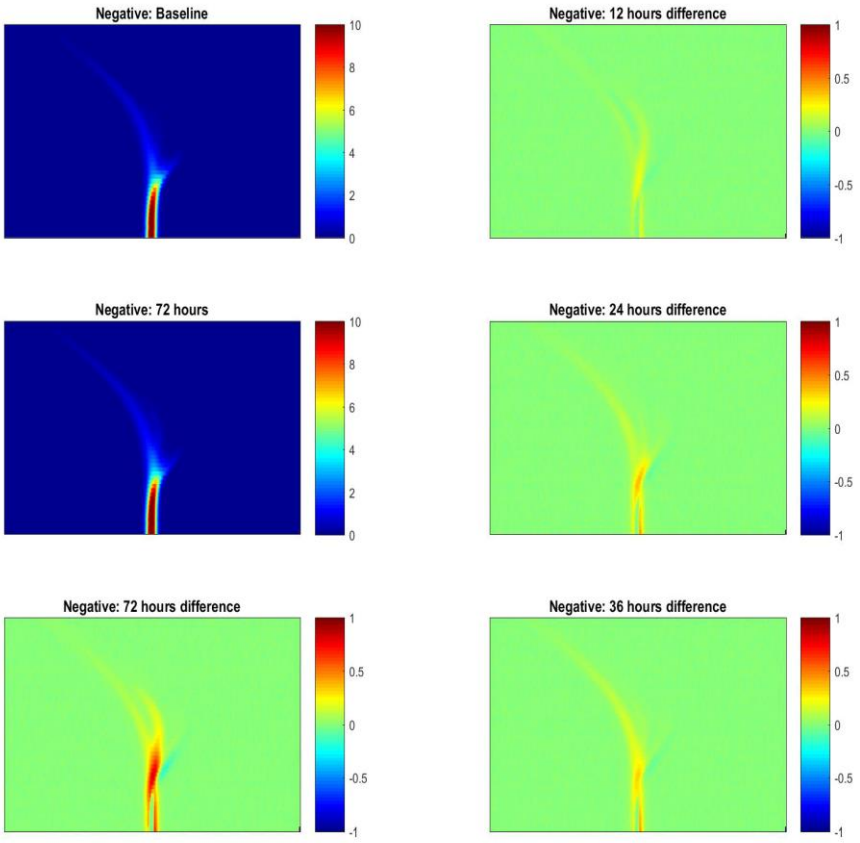


Figure 4.2b. Negative matrices, difference from baseline with time. Top left: FAIMS spectrum at time zero. Middle left: FAIMS spectrum at 72 hours. Bottom left: Difference between spectrum at 72 hours and baseline. This shows a predominantly positive difference spectrum indicating an increase in VOCs. Right hand panels show the difference at 12, 24 and 36 hours from time 0, showing a consistent pattern, developing with time. All matrices averaged over all patients.

To try and identify the source of this variation in the matrices, the average relative pairwise differences for all patients across each of the 14 matrices at each time point was plotted for both positive and negative matrices. This is shown in figure 4.3a for positive matrices and figure 4.3b for negative matrices. Each 14x14 block corresponds to every pairwise difference between the 14 matrices at each time point i.e. Time point 0, matrix 1 vs time point 0, matrix 1/2/3/4 etc. The structure of the block suggests that there is significant variation within the fourteen matrices at any given time point. The difference between the first matrix from each time point and the subsequent matrices from the same time point grows with matrix number. This variation from baseline also grows with increasing time point from time point 0, as shown above.

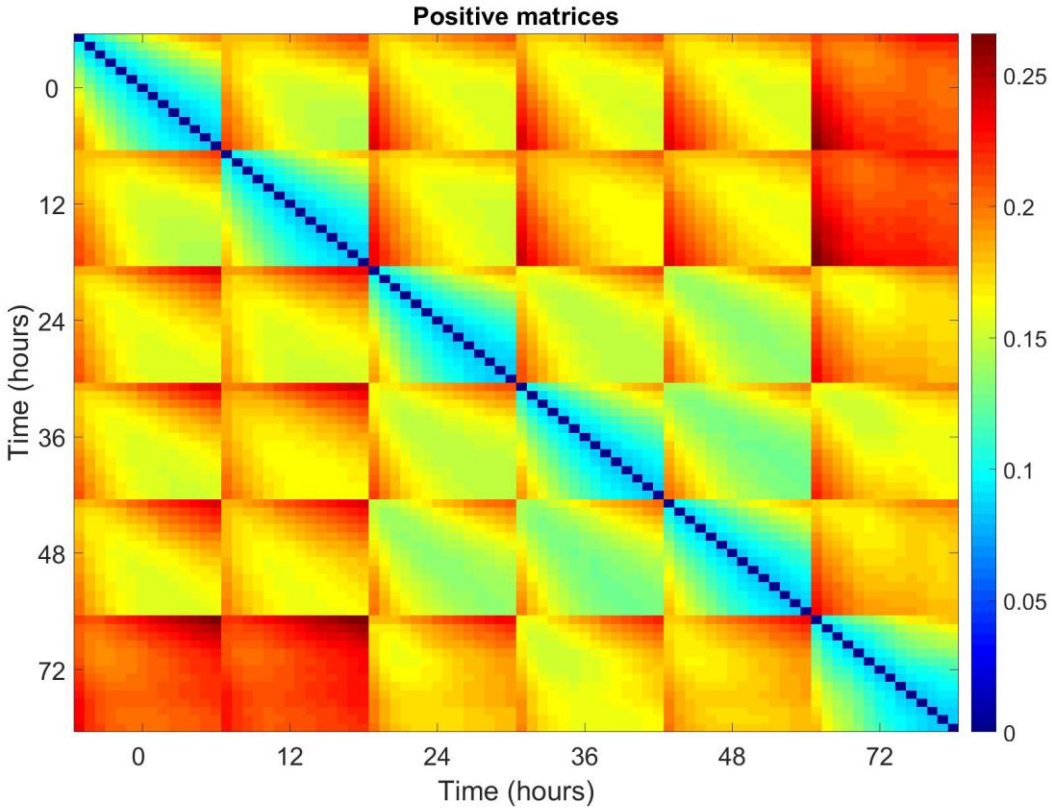


Figure 4.3a. Pairwise relative differences for all pairs of positive matrices averaged across all patients. Blocks correspond to the labelled times, with 14 x 14 elements corresponding to the associated matrices.

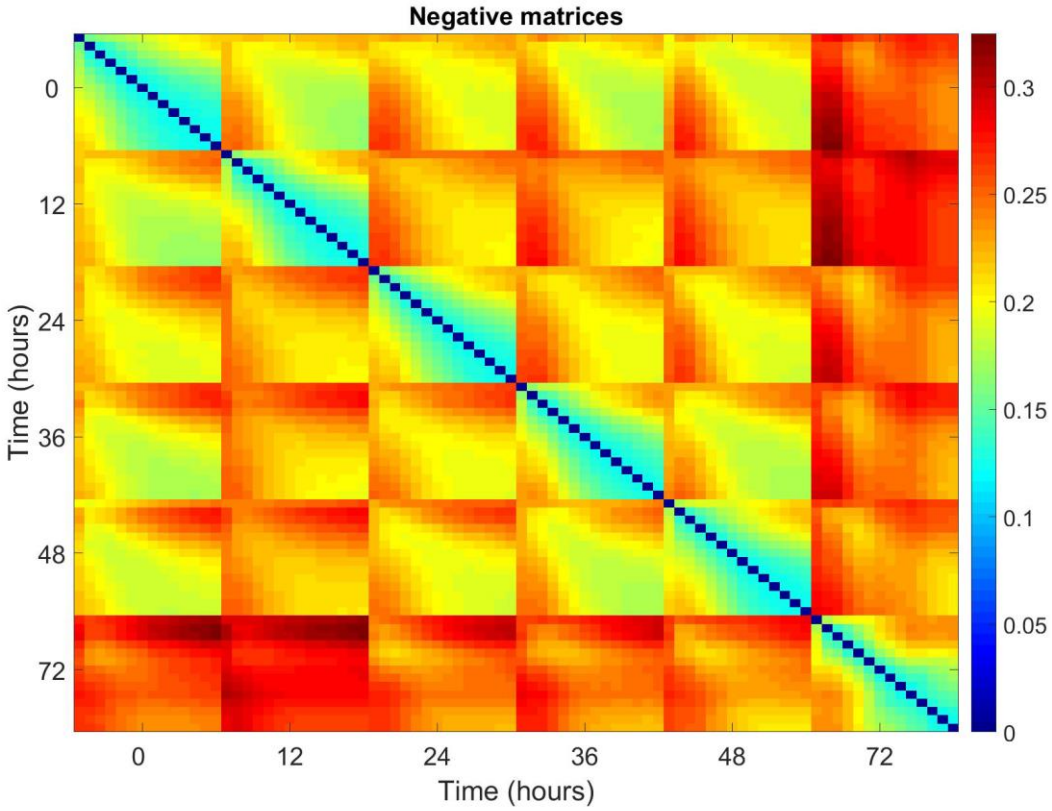


Figure 4.3b. Pairwise relative differences for all pairs of negative matrices averaged across all patients. Blocks correspond to the labelled times, with 14 x 14 elements corresponding to the associated matrices.

To investigate this further, the relative variation in FAIMS matrix between the 14 matrices from each time point was calculated for both positive and negative matrices.

Mathematically this was done by:

$$\text{Variation } t,n = \frac{\Sigma p\ Vp,t,n}{P}$$

Where: $$Vp,t,n = \frac{\Sigma c \Sigma s |Mp,t,n,c,s - \Sigma n Mp,t,n=1,c,s|}{\Sigma c \Sigma s |Mp,t,n=1,c,s|}$$

Figures 4.4a and 4.4b show the relative variation in averaged FAIM matrices for all patients for positive and negative matrices, respectively, compared to increasing matrix number. These show a monotonic increase in relative variation for both positive and negative matrices at all time points, with increasing matrix number for that time point.

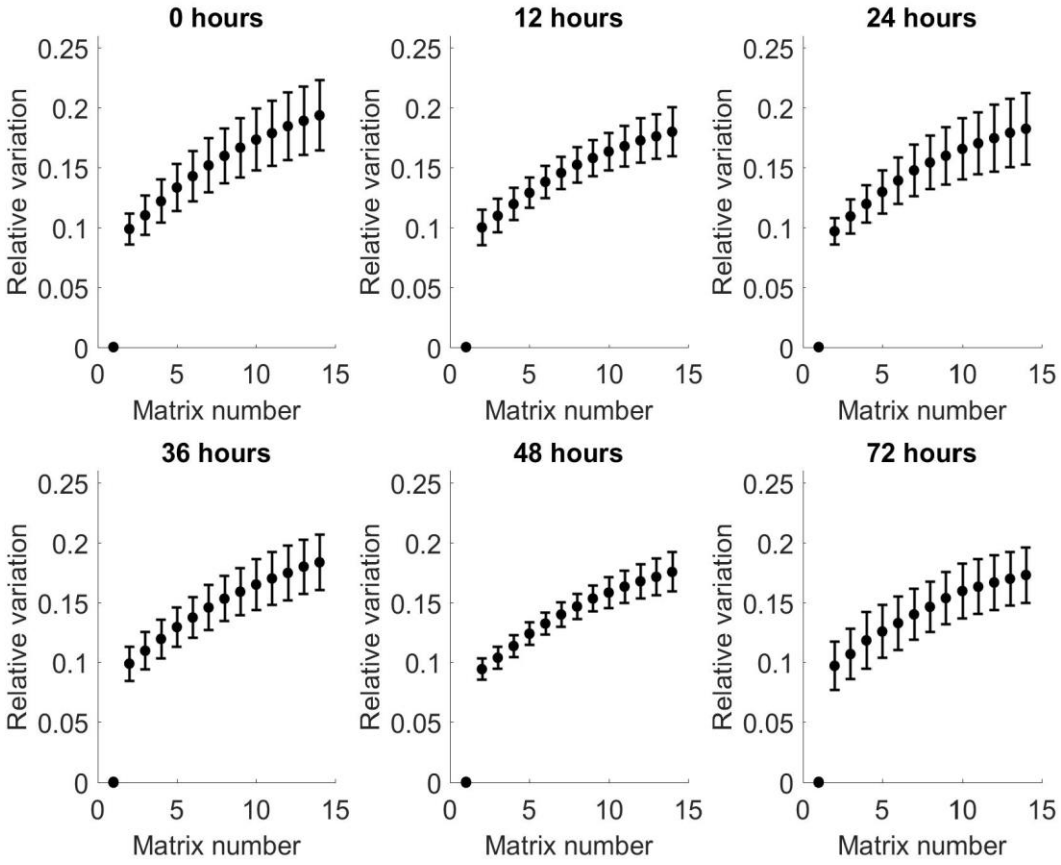


Figure 4.4a. Relative variation from baseline of arithmetic mean of all patients as a function of matrix number for each time point for positive matrices. Bars show standard deviation.

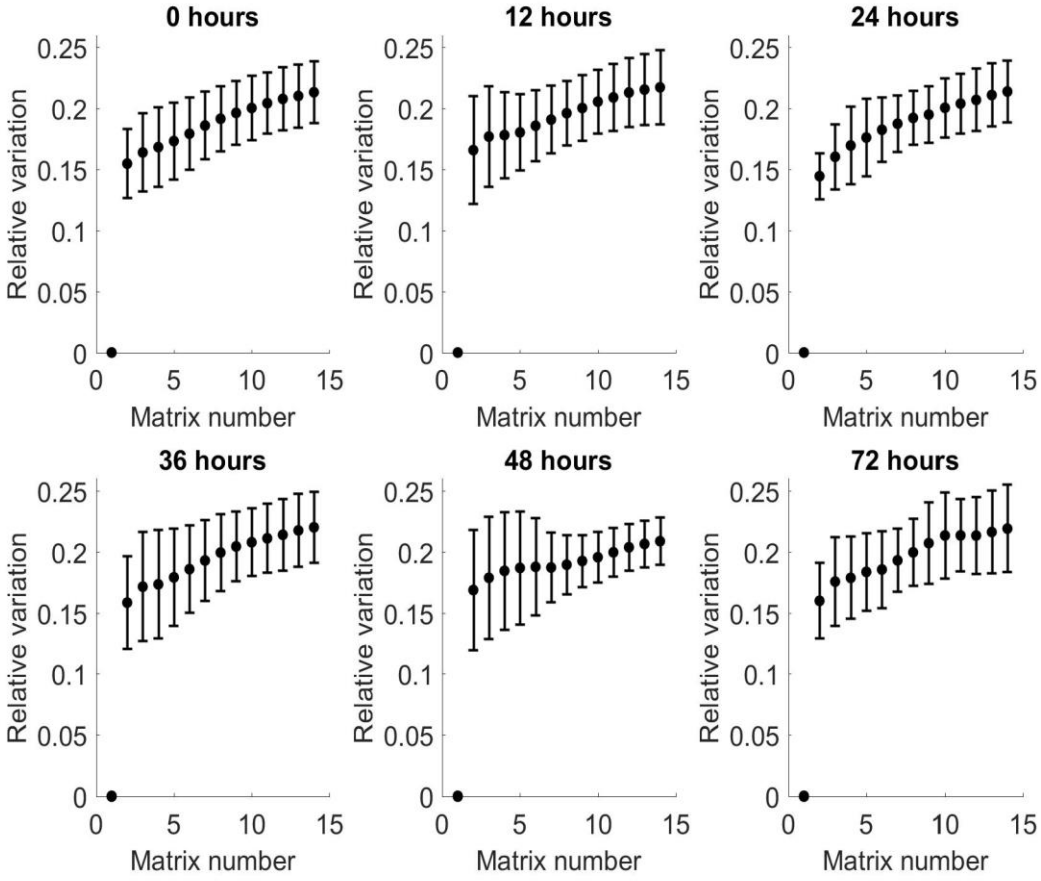


Figure 4.4b. Relative variation from baseline of arithmetic mean of all patients as a function of matrix number for each time point for negative matrices. Bars show standard deviation.

This increase in relative variation, with increasing matrix number for each time point, was reflected in the FAIMS spectra, as was seen earlier with the relative variation of matrix structure seen with increasing time point. Figure 4.5a and 4.5b show the differences between the average matrices for all patients from matrix 1, time point 0, compared to matrix 14, time point 0 for both positive and negative matrices. Matrices 5, 8 and 11 from time point 0 show the consistent pattern of change with increasing matrix number. There is a predominantly negative difference in spectra between matrix 1 and 14 at each time point. This indicates that there is a loss of VOCs with increasing matrix number at each same time point. These findings are also seen in the negative matrices.

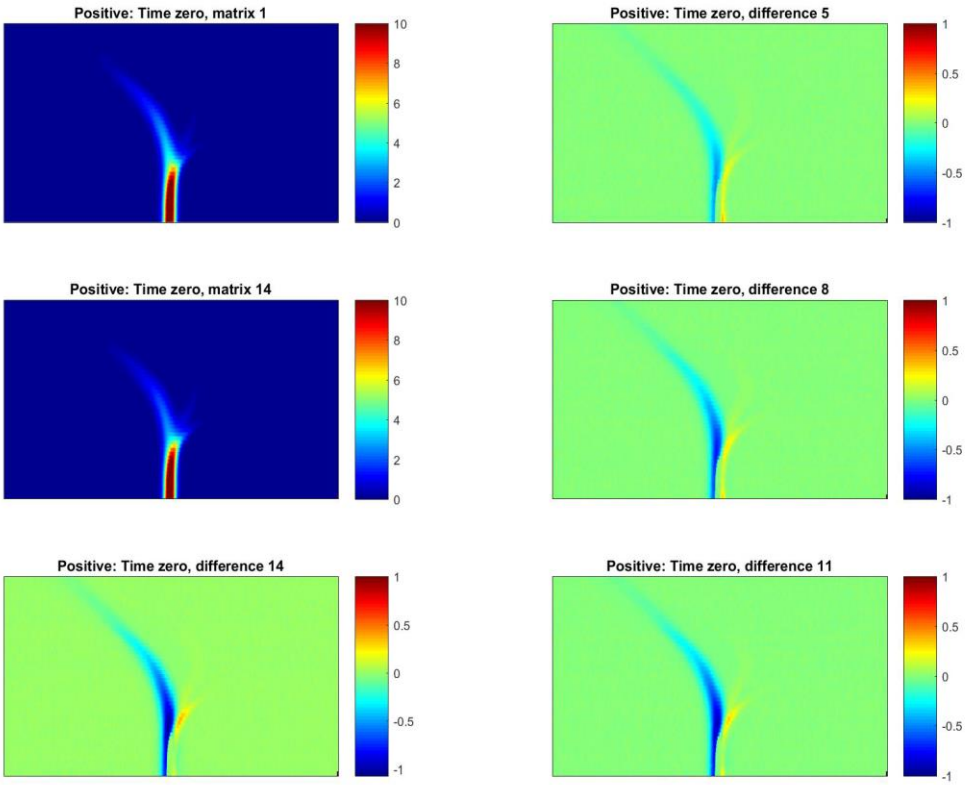


Figure 4.5a. Differences from baseline with increasing matrix number for positive matrices averaged over all subjects at t=0. Top left: Matrix 1, time 0. Middle left: matrix 14, time 0. Bottom left: difference between spectrum 1 and spectrum 14. The predominantly negative difference spectrum indicates a loss of VOCs. Right hand panels show the difference for matrices 5, 8 and 11 from matrix 1, showing a consistent pattern developing over time.

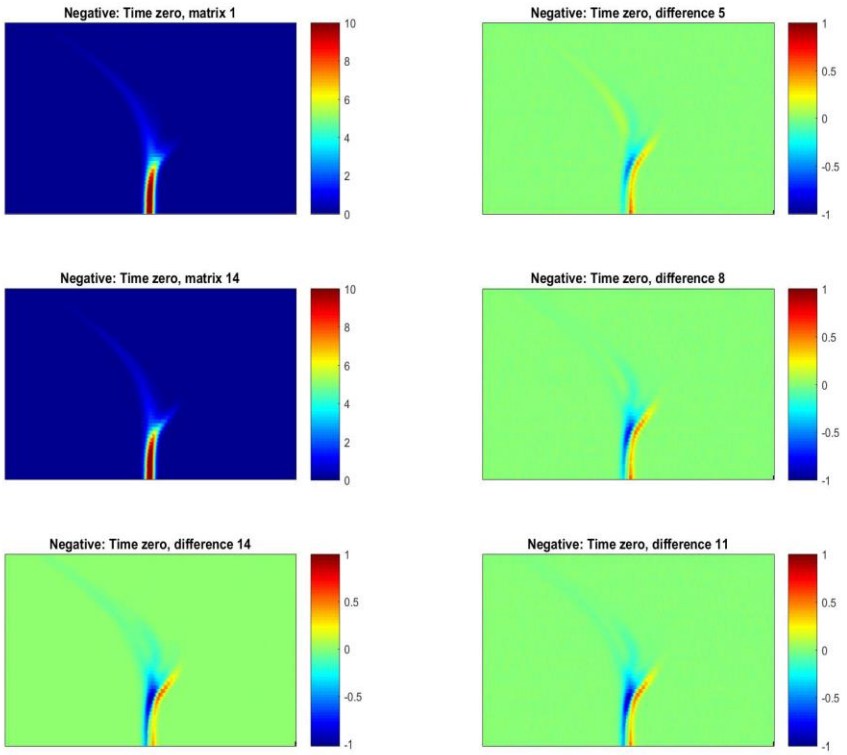


Figure 4.5b. Differences from baseline with increasing matrix number for negative matrices averaged over all subjects at t=0. Top left: Matrix 1, time 0. Middle left: matrix 14, time 0. Bottom left: difference between spectrum 1 and spectrum 14. The predominantly negative difference spectrum indicates a loss of VOCs. Right hand panels show the difference for matrices 5, 8 and 11 from matrix 1, showing a consistent pattern developing over time.

Relative variation in FAIMS spectra, and consequently VOC profile, not only increases with increasing room termpeature exposure time prior to freezing, but also with increasing matrix number for each time point. This suggests that VOC profiles are altering, not only with increased exposure to room temperature before freezing, but also to increasing atmospheric exposure on thawing. A subset of positive and negative matrices were analysed to see if there was any effect from using earlier, "fresher" matrices for each time point.

Subsets of: the first matrix; the means of the first three matrices and all fourteen matrices from each patient at each time point were analysed. There was no qualitative difference in relative variation over the various time points when using a subset of matrices. The mean variation with time for the mean of all fourteen matrices per subject per time point gave a Spearman's rank correlation coefficient of ρ0.94, p = 0.017 for positive matrices, and ρ0.83, p = 0.058 for negative matrices. When a subset of the first three matrices was used; ρ0.66, p = 0.175 for positive matrices and ρ0.66, p = 0.175 for negative matrices. When only the first matrix for each patient at each time point was used; ρ0.43, p = 0.41 for positive matrices ρ0.66, p = 0.175 for negative matrices. These data are shown in figure 4.6a and 4.6b.

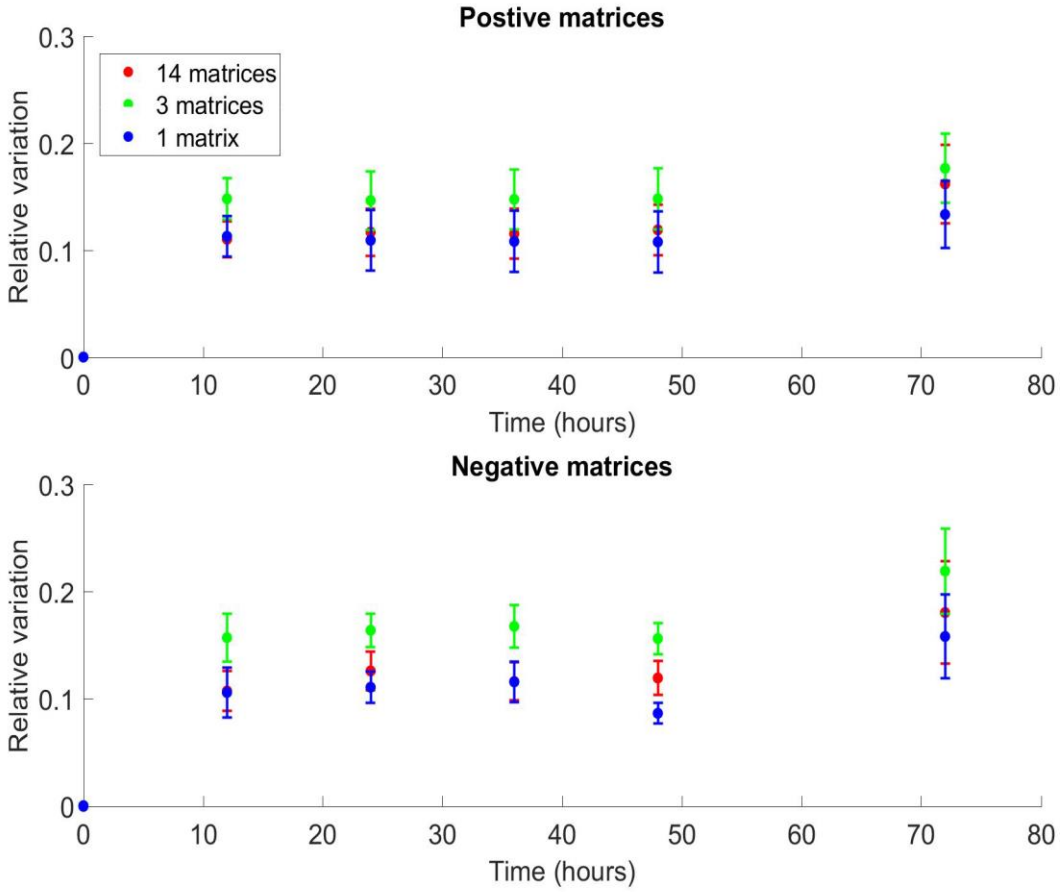


Figure 4.6a. Correlation of mean variation of positive FAIMS matrices from mean of matrices from t=0. Red: Athrimetic mean of all 14 matrices from each patient at each time point. Green: Artithmetic mean of first 3 matrices from each patient at each time point. Blue: Variation calculated using only matrix 1 from each patient at each time point.

Figure 4.6b. Correlation of mean variation of negative FAIMS matrices from mean of matrices from t=0. Red: Athrimetic mean of all 14 matrices from each patient at each time point. Green: Artithmetic mean of first 3 matrices from each patient at each time point. Blue: Variation calculated using only matrix 1 from each patient at each time point.

4.3.2. Relative variation in Total Ion Count

The relative variation in the total ion counts from the urine samples was also calculated as a function of time, as described in section 3.3.2.3. This was done for positive and negative FAIMS matrices.

The total ion count was found to increase with time for both positive and negative FAIMS matrices. This is shown in figure 4.7a for positive matrices and 4.7b for negative matrices. Using Spearman's rank correlation coefficient to test for a monotonic relationship between time and variation in mean total ion count from baseline, gave $\rho 0.94$, $p = 0.017$ for positive matrices, and $\rho 0.94$, $p = 0.017$ for negative matrices. When all patients were combined as a single time series, this gave $\rho 0.25$, $p = 0.009$ for positive matrices, and $\rho 0.27$, $p = 0.004$ for negative matrices.

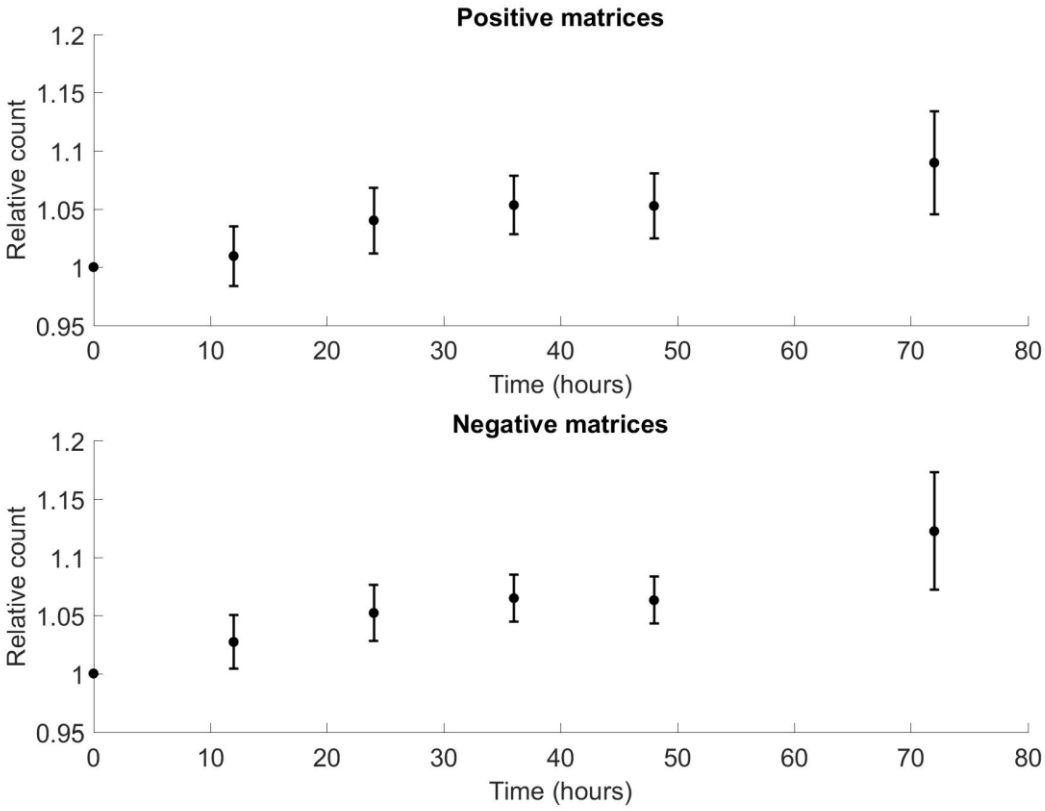


Figure 4.7a. Variation in relative ion count for positive matrices from mean of matrices at t=0. Bars show standard error. Correlation of mean ion count with time: Spearman ρ0.94, p = 0.017; correlation of all points with time: Spearman ρ0.25, p = 0.009. Plateau phase seen between 12 and 48 hours.

Figure 4.7b. Variation in relative ion count for negative matrices from mean of matrices at t=0. Bars show standard error. Correlation of mean ion count with time: Spearman ρ0.94, p = 0.017; correlation of all points with time: Spearman ρ0.27, p = 0.004. Plateau phase seen between 12 and 48 hours.

Given the finding that there is an increase in variation when comparing sequential matrices from the same sample at a given time point described in the previous section, an analysis was run on the total ion count variation for sequential matrices from each time point. This analysis showed that the relative ion count reduced monotonically for positive matrices, with increasing matrix number at the same time point, and increased transiently for negative matrices before also reducing. These results are shown in Figure 4.8a and 4.8b.

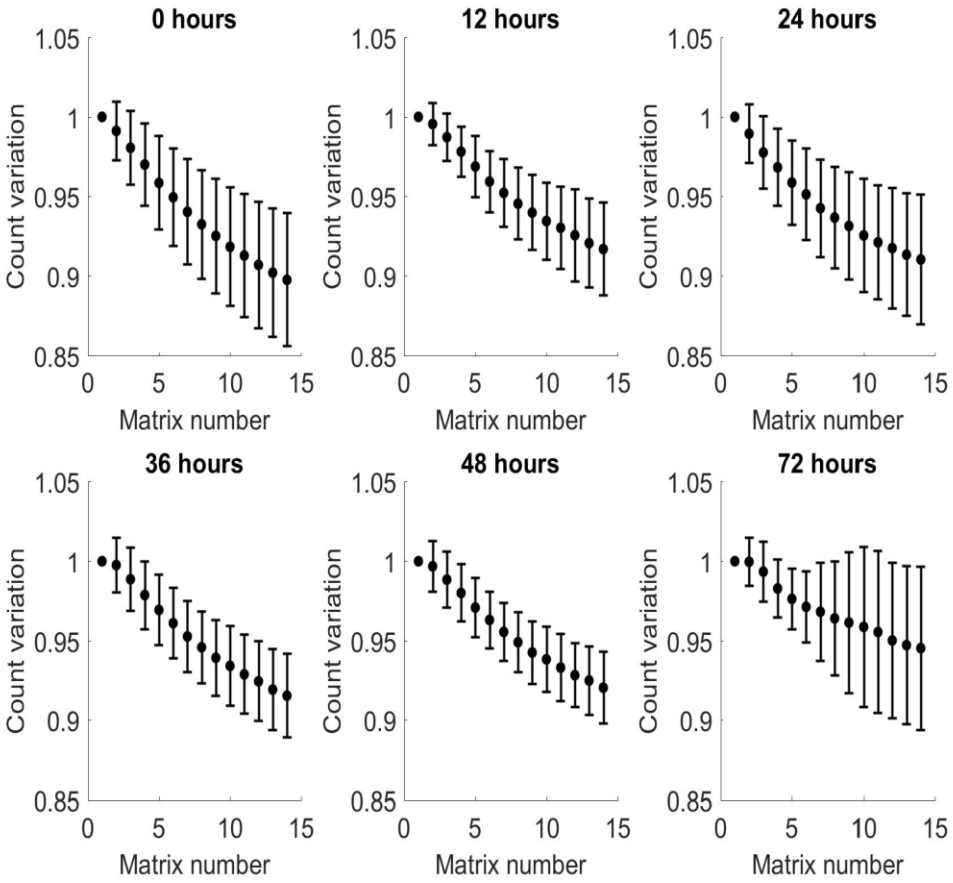


Figure 4.8a. Relative variation of ion count from baseline of arithmetic mean of all patients as a function of matrix number for each time point for positive matrices. Bars show standard deviation.

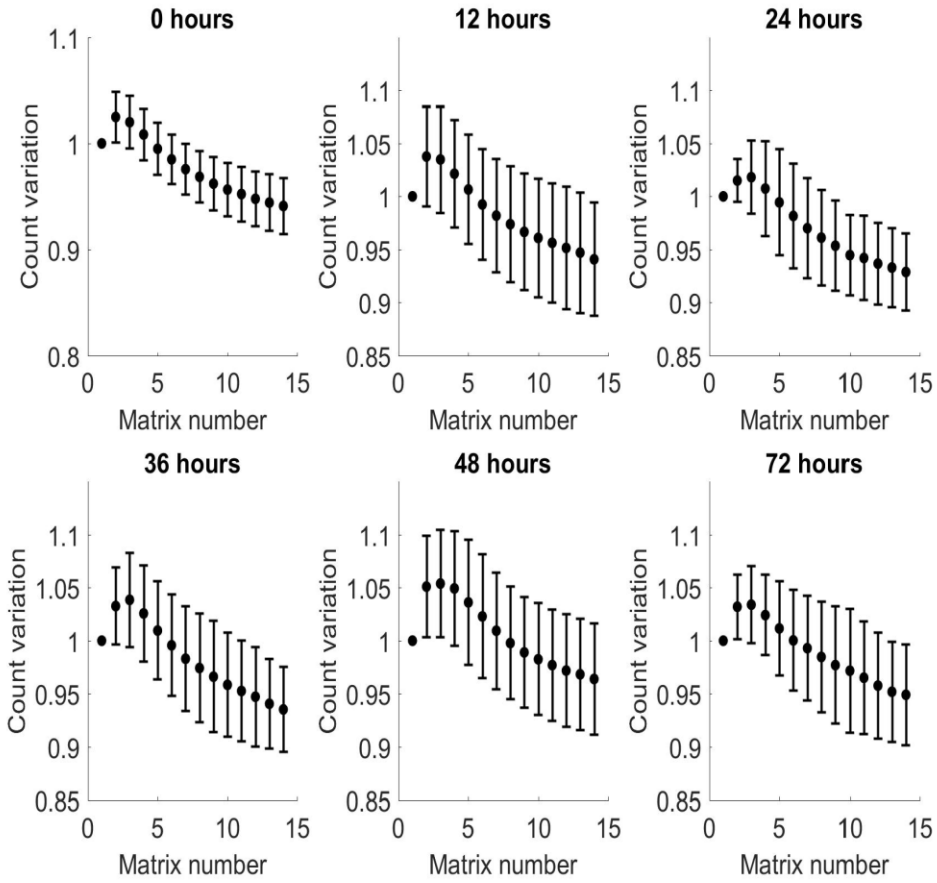


Figure 4.8b. Relative variation of ion count from baseline of arithmetic mean of all patients as a function of matrix number for each time point for negative matrices. Bars show standard deviation.

As the relative ion count variation increases with time before the sample is frozen, but decreases with matrix number after the sample is thawed, subsets of the matrices were analysed for each patient at each time point to determine if using earlier matrices gave different results.

Subsets of: the first matrix; the means of the first three matrices and all fourteen matrices from each patient at each time point were analysed. There was no qualitative difference in relative variation over the various time points when using a subset of matrices. The mean variation with time for the mean of all fourteen matrices per subject per time point, gave a Spearman's rank correlation coefficient of ρ1.0, p = 0.003 for positive matrices, and ρ1.0, p = 0.003 for negative matrices. When a subset of the first three matrices was used; ρ0.94, p = 0.17 for positive matrices, and ρ0.83, p = 0.058 for negative matrices. When only the first matrix for each patient at each time point was used; ρ0.77, p = 0.103 for positive matrices ρ0.77, p = 0.103 for negative matrices. These data are shown in figure 4.9a and 4.9b.

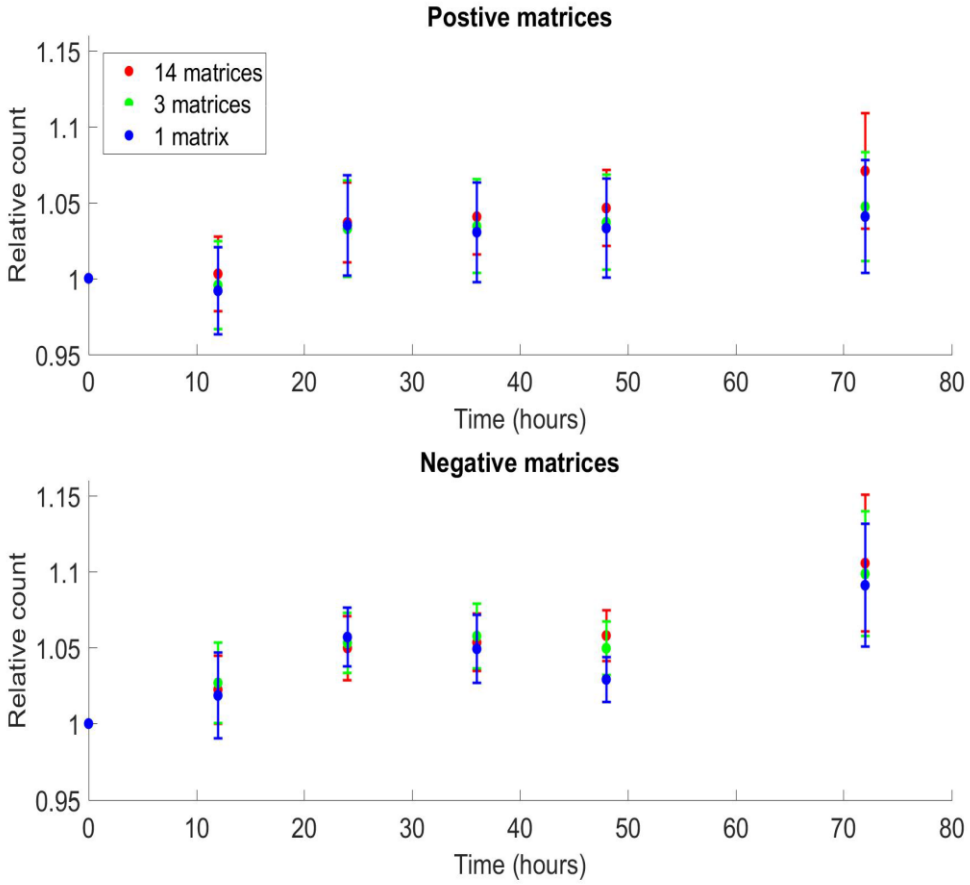


Figure 4.9a. Correlation of mean mean relative ion count variation of positive matrices from t=0. Red: Athrimetic mean of all 14 matrices from each patient at each time point. Green: Artithmetic mean of first 3 matrices from each patient at each time point. Blue: Variation calculated using only matrix 1 from each patient at each time point.

Figure 4.9b. Correlation of mean relative ion count variation of negative matrices from t=0. Red: Athrimetic mean of all 14 matrices from each patient at each time point. Green: Artithmetic mean of first 3 matrices from each patient at each time point. Blue: Variation calculated using only matrix 1 from each patient at each time point.

4.4 Discussion

Analysis of the patterns of VOCs found in the urine of healthy subjects, in response to prolonged storage at room temperature prior to freezing, shows that the variation within the FAIMS matrices, and hence the VOCs, increases with prolonged exposure to room temperature, despite storage in a sealed container. This pattern was uniform across all subjects, suggesting that this is an inherent property of the VOCs themselves. The presence of a plateau within this increase in variation, between 12 and 48 hours, is interesting. This could indicate that, after an initial degradation period, only minimal further alteration to the VOC profile occurs. This may represent the VOCs reaching a "steady state", and that it is only with exposure to room temperature beyond 48 hours that further degradation of VOCs occurs. The variation within the spectra was shown to increase with time, however, within each time point, the variation also increased in sequential matrices, despite an overall loss of the number of ions seen. This suggests that the samples were degrading to produce a more diverse pattern, but a reduced overall number of ionised VOCs.

The cause of this degradation is unclear. All samples were aliquoted and sealed immediately upon collection. This reduces the likelihood of outside contaminants, but does not completely eliminate it. Urine was previously held to be a sterile substance, although this has recently been disproved (243). Despite none of our subjects had genitourinary disease, the presence of bacterial contamination from the urinary tract cannot be excluded, and, cannot be controlled for beyond

selecting healthy individuals. Bacterial contamination from the environment is a possibility, with potential introduction during the collection of the samples, although exposure of the samples to the atmosphere was kept to a minimum, and sterile universal bottles used. Any universal bottle which had a loose or absent lid when removed from its storage bag was discarded. Bacteria could potentially have been introduced during the sample analysis, although, exposure to atmospheric air was kept to a minimum, with samples being defrosted with the bottle caps secured. All samples were defrosted at room temperature and only re-exposed to atmospheric air once they were thawed and ready for analysis. This time was kept to a minimum, but, as before, outside contamination cannot be completely excluded.

Given the consistent pattern of sample degradation, it suggests that any source of outside bacterial contamination occurred across all samples. Freezing the samples to -80°C would not kill all potentially contaminating bacteria, so if contamination were present then the bacteria could have lain dormant within the samples and metabolic processes restarted as the samples were thawed. Once again, this is unlikely given the healthy nature of our subjects but cannot be excluded.

The increase in variation of VOCs with time was also reflected in an increase in total ion count within the samples with time exposed to room temperature prior to freezing. This suggests that the chemicals and VOCs within the sample are undergoing a degradation process, which results in several smaller molecules arising from the original molecules. The mechanism by which this occurs is unclear,

it could represent the activity of enzymes present in the urine, or other catalytic substances present within the urine, which allow the degradation and alteration of chemicals and VOCs, found within the samples, to multiple smaller compounds, and hence lead to an increase in VOC variation and total ion count.

The observation that VOC variation increases and total ion count decreases with increasing matrix number for each time point is interesting. This suggests that VOC degradation continues upon thawing of the samples, and, with prolonged exposure to atmospheric conditions after thawing. This results in a greater relative variation of the VOC profile from the first matrix of each time point, to matrix fourteen of each time point, but an overall reduction in ion count number. This variation was consistent across all subjects and time points. The finding that total ion count reduces with increasing matrix number for each time point could suggest that the ions are being consumed in the process of producing new VOC degradation products, and that the ions themselves may be driving the degradation process.

From a practical point of view, this study has far reaching effects for any further work which is to be performed using VOC analysis, as it affects sample collection and storage. Given the rapid degradation of VOC profile which occurs between 0 and 12 hours, and then the plateau which occurs between 12 and 48 hours, there are two potential strategies. The first is that only absolutely fresh samples should be used, and they must be frozen immediately to prevent degradation. This has logistical implications for sample collection in a research environment and may

not always be achievable, particularly if the research subjects are uncomfortable with providing samples in a hospital or research setting. The second option would be to allow subjects to produce their samples at their convenience and return the samples, provided this occurs within 12-48 hours of the urine being passed into the bottle. This would rely on a high level of patient education and compliance. This would also require that any freshly produced urine should be stored at room temperature, so that all samples are frozen at the same time, to ensure that they are all exposed to atmospheric temperature for an equal amount of time.

As an example from the main experiment of this thesis, the urine collected from the CRC patients, their relatives and spouses, was collected in a very heterogenous fashion. Some samples were provided on the day of surgery and were frozen with 30 minutes of production. Others were done the same morning, and some the night before. Some specimens were returned via hospital courier, which would add several hours of exposure to atmospheric temperature. At the extreme end of the experiment, some samples were returned via the postal service, which, although first class postage was used, could result in a 48-72 hour exposure to atmospheric temperature. Although the vast majority of samples would have been returned and frozen within the 12-48 hour plateau phase of the degradation curve. This means that the results of the main experiment, detailed in the next chapter, are valid within the context of sample degradation. No samples were exposed to atmospheric temperature for greater than 72 hours.

This wide ranging spectrum of times for storage at atmospheric temperature means that some samples will have been further along the degradation curve than others. This could result in skewing of results due to overly degraded samples, however, the majority of samples were returned within the 12-48 hour plateau phase and the numbers of samples from the two extremes of the time spectrum were equally distributed this effect should be negated.

Given the uniform way in which the samples appear to degrade with exposure to room temperature, there is the possibility that a "degradation algorithm" could be calculated, that would allow predicted of the original VOC profile, if the time a sample was exposed to atmospheric air was known. This again would require a high level of subject compliance, to accurately write the time and date the sample was produced on the collection bottle. It could also require good documentation at the tissue bank to accurately record when samples were frozen. This would allow the time the sample was exposed to room/atmospheric temperature to be calculated and the necessary calculations performed. This calculation is beyond the scope of this thesis, but is an interesting consideration for VOC research as it moves forward.

This experiment highlights how little is known of the natural history of VOCs in health and disease. This experiment was conducted on healthy subjects, and there is currently no available research into the degradation of VOCs in diseased subjects. It is not known if the degradation follows a similar pattern, as in healthy subjects. Furthermore, this study had a sample size of only 20. Further, larger

scale studies, potentially over longer time periods and with more interim time points, are needed, to allow a full mapping of the degradation patterns of VOCs in urine with prolonged exposure to atmospheric temperature prior to freezing for subsequent analysis.

CHAPTER 5

Urinary VOC profiling of colorectal cancer patients, their first degree relatives and co-habitors

5.1. Introduction

This experiment was aimed at characterising the urinary VOC profiles of colorectal cancer patients, prior to surgery or chemo/radiotherapy, in addition to their first degree relatives and spouses/co-habitors.

The aim of this experiment was, in the first place, to confirm that CRC patients produce a unique VOC profile, which is distinct from those who share genetic traits, and those who share an environment. Previous studies into VOC profiling of CRC patients had used unrelated controls who also did not share an environment with the subjects.

A further aim of the study was to determine whether either of the control groups had a VOC profile which more closely resembled that of the CRC group. In other words, could we determine if environment or genetics appeared to be a greater contributor towards the VOC profile being detected in CRC patients.

The characterisation of the urinary VOC profiles was initially planned to be conducted on a Lonestar FAIMS machine at the University of Warwick, however, due to technical issues with the machine and recurrent delays, the decision was made to approach the company which manufactured the FAIMS machine, Owlstone, directly, to perform the analysis. This was performed on a variant of the FAIMS machine, which had a liquid chromatography column and time of flight mass spectrometer attached to the main FAIMS machine.

5.2 Methods

CRC patients were recruited as described in section 3.2.1. Relatives and spouses of the CRC patients were recruited as described in section 3.2.2. Only first degree relatives were approached to preserve as much of the genetic "signal" as possible. This included siblings and children. Children still co-habiting with the CRC patients were excluded as they would represent both genetic and environmental controls and could lead to confounding. Sample collection and storage was performed as described in sections 3.2.3. The samples were frozen at -80°C as soon as possible after their receipt. The results of Chapter 4 show that the time frame for sample collection (0-72 hours) will not cause a significant effect on the results gathered here, as while there is alteration of the signal with increasing sample exposure to atmospheric temperature, it is not enough to sigficiantly affect the results gathered. They were transferred to Owlstone, Cambridge in dry ice and again stored frozen at -80°C. Samples were thawed and analysed using the LC-FAIMS-MS hybrid (Owlstone, UK), as described in section 3.3.1. Data processing and statistical analysis were performed as described in sections 3.3.1.2a and 3.3.1.2.b.

5.3. Results

72 CRC patients were recruited, in addition to 61 first degree relatives and 56 spouses. Samples were returned by 56 pre-treatment CRC patients, 37 first degree relatives and 45 spouses.

The mean ages of the three cohorts were 65.4 years (SD 11.5), 50 years (SD 14.1) and 60.7 years (SD 12.1) respectively. Normal distribution of ages was confirmed using Shapiro-Wilk testing. Analysis of Variance (ANOVA) testing and post-hoc Tukey's honest significance difference (HSD) test showed no statistically significant differences in ages between CRC patients and their spouses. However, there was a significant difference between both CRC and first degree relatives, and spouses and first degree relatives, $p < 0.01$. This is due to presence of both siblings and children of the cancer patients in the cohort. This is an unavoidable consequence of recruiting children of the cancer patients.

There were 33 males and 23 females with CRC in the final cohort, this ratio, was expectedly, reversed in the spouse cohort, with 15 males and 30 females. The male to female ratio in the relative cohort was 17:20. The male: female distribution was analysed using the Chi squared test, and found to be significantly different, chi squared statistic 10.3, $p = 0.016$. Again this is to be expected, given the male predominance in CRC.

The average cigarette consumption per day was 1.5 (SD 4.2) for CRC patients, 2.5 (SD 5.3) for first degree relatives, and 1.6 (SD 5.7) for spouses. There was no statistically significant difference using ANOVA testing, $p = 0.26$.

Average alcohol consumption (units per week) was 8.8 (SD 11.6) for CRC patients, 7.3 (SD 8.1) for first degree relatives and 6.7 (SD 10.1) for spouses. There was no statistically significant difference using ANOVA testing, p = 0.94.

Mean BMI for the CRC cohort was 27.5 (SD 5.2), 25.5 (SD 3.8) for the first degree relatives and 26.7 (SD 4.8) for the spouses. There was no statistically significant difference using ANOVA testing, p = 0.45.

The Duke's stage of the CRC patients was as follows: A = 8 (14.2%), B = 17 (30.4%), C1 =20 (35.7%), C2 =9 (16.1%). The anatomical distribution of the CRC subjects was: right sided = 24 (42.8%); left sided = 17 (30.4%) and rectal = 15 (26.8%). The referral pathways that the CRC patients were detected through were: Screening programme = 18 (32.1%), 2 week wait pathway = 31 (55.4%), and other (including routine, emergency and incidental findings) =7 (12.5%).

The demographics of the recruited subjects can be found in figure 5.4.

No CRC patients, relatives or spouses had received recent courses of antibiotics. The CRC subjects had not undergone colonoscopy, and hence consumed bowel preparation medication, within the previous 2 weeks.

3 CRC patients had a first degree relative (sibling or parent) with a history of CRC, these relatives were all diagnosed over the age of 60, meaning that all of our patients were sporadic CRC patients.

Group	Pre-treatment CRC	Relative	Spouse	P value
Samples	56	37	45	
Mean age (SD)	65.4 (11.5)	50.0 (14.1)*	60.7 (12.1)	* <0.01
Sex (M:F)	33:23	17:20	15:30	0.016
Number of cigarettes smoked per day (SD)	1.5 (4.2)	2.5 (5.3)	1.6 (5.7)	0.26
Alcohol units per week (SD)	8.8 (11.6)	7.3 (8.1)	6.7 (10.1)	0.94
Mean BMI (SD)	27.5 (5.2)	25.5 (3.8)	26.7 (4.8)	0.45
Dukes stage (%)				
A	8 (14.2%)	-	-	
B	17 (30.4%)	-	-	
C1	20 (35.7%)	-	-	
C2	9 (16.1%)	-	-	
Site (%)				
Right	24 (42.8%)	-	-	
Left	17 (30.4%)	-	-	
Rectal	15 (26.8%)	-	-	
Referral Route (%)				
Screening	18 (32.1%)	-	-	
2WW	31 (55.4%)	-	-	
Other	7 (12.5%)	-	-	

Table 5.1 Demographic data from recruited pre-treatment CRC patients, their first degree relatives and co-habitors/spouses.

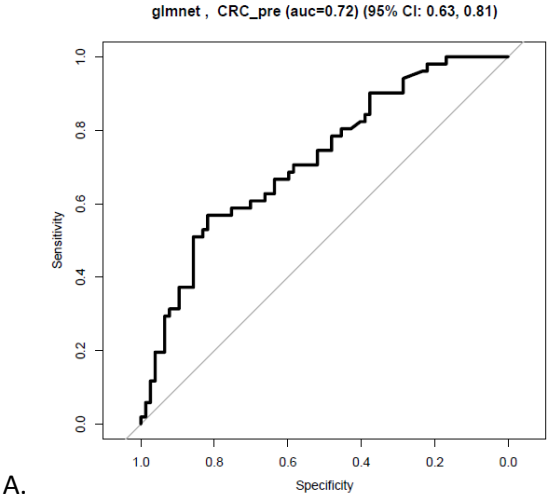
The LC-FAIMS-MS data was analysed as described in section 3.3.1.2. The relevant subset of samples was extracted, and the different groups defined. 5-fold cross-validation was then used to assess classification accuracy across these groups, using three different multi-class classifiers: sparse logistic regression, Support Vector Machine, Random Forest. This analysis generated outputs of one-vs-all ROC curves i.e. comparing a single group vs all other groups, for example CRC vs relatives and spouses, relatives vs CRC and spouses, spouses vs CRC and relatives. Other results generated included the Area-Under-Curve (AUC) statistic, sensitivity/specificity values, which were selected automatically to be maximally similar given the ROC curve, and a p-value comparing the result to that expected for random chance (AUC=0.5), using a Wilcoxon rank-sum test.

This analysis showed that the LC-FAIMS-MS technology was able to distinguish pre-treatment CRC subjects from relatives and spouses as follows:

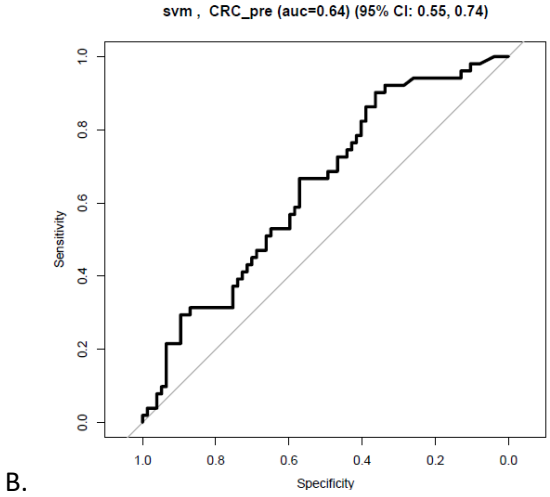
Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.63 (0.48 – 0.76)	0.64 (0.52 – 0.74)	0.72 (0.63 – 0.81)	9	0.000256*
Support vector Machine	0.59 (0.44 – 0.72)	0.58 (0.47 – 0.77)	0.64 (0.55 – 0.74)	9	0.0617
Random Forrest	0.69 (0.54 – 0.81)	0.69 (0.57 – 0.79)	0.71 (0.62 – 0.8)	9	0.00058*

The Receiver Operator Curve plots for these 3 analyses can be found in figure 5.1.

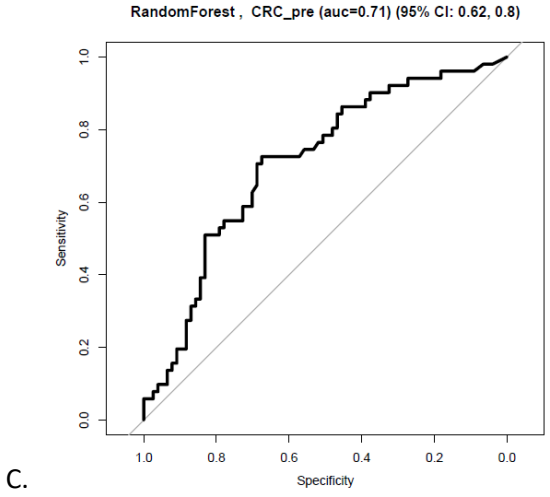
The positive predictive value for the Sparse logistic regression analysis was 54%, and negative predictive value 46%. The positive predictive value of the Random Forrest analysis was 60%, and negative predictive value 76%.



A.



B.



C.

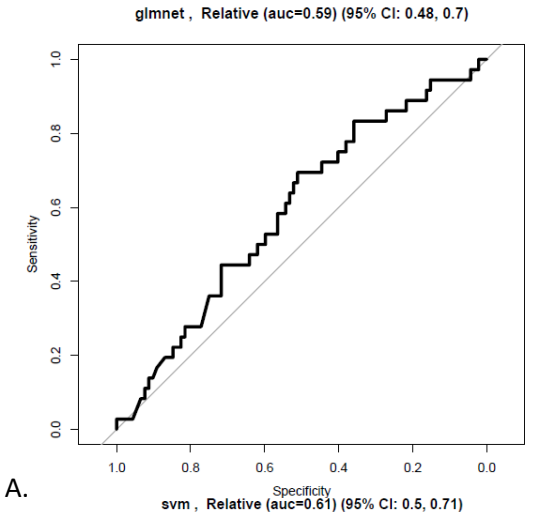
Figure 5.1. Receiver Operator Curve plots for statistical pipelines used to distinguish pre-treatment CRC patients from the relative and spouse/co-habitor control groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

When the relative and spouse groups were individually cross validated against a combination of the other 2 groups e.g. relatives vs CRC and spouses, spouses vs CRC and relatives, then the following, non-statistically significant, results were achieved:

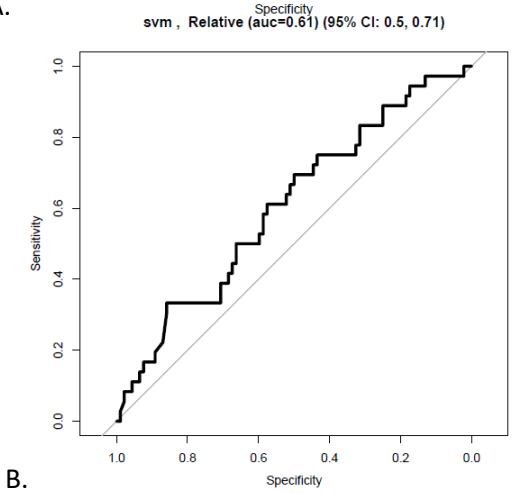
Relatives

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.56 (0.38 – 0.72)	0.57 (0.46 – 0.67)	0.59 (0.48 – 0.7)	9	0.933
Support vector Machine	0.58 (0.41 – 0.74)	0.59 (0.48 – 0.69)	0.61 (0.5 – 0.71)	9	0.569
Random Forrest	0.56 (0.38 – 0.72)	0.55 (0.45 – 0.66)	0.62 (0.51 – 0.73)	9	0.287

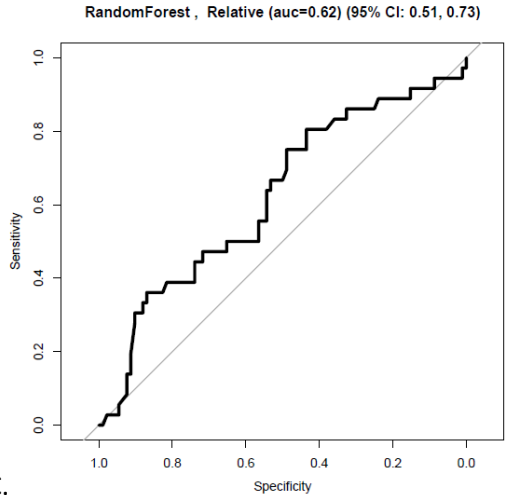
The Receiver Operator Curve plots for these 3 analyses can be found in figure 5.2.



A.



B.



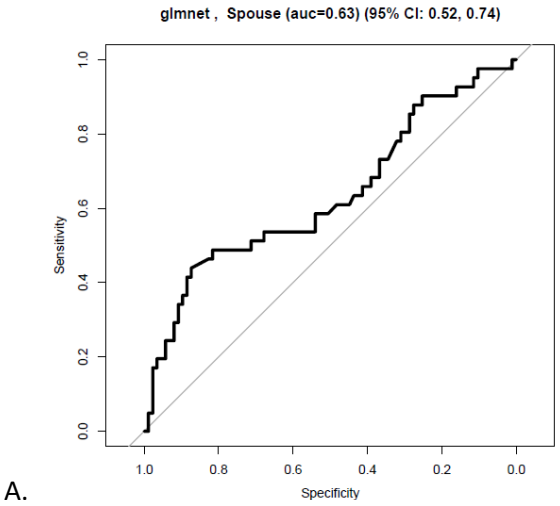
C.

Figure 5.2. Receiver Operator Curve plots for statistical pipelines used to distinguish relatives from the pre-treatment CRC patients and spouse/co-habitor groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

Spouses

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.54 (0.37 – 0.69)	0.54 (0.43 – 0.65)	0.63 (0.52 – 0.74)	9	0.133
Support vector Machine	0.51 (0.35 – 0.67)	0.52 (0.41 – 0.63)	0.49 (0.37 – 0.6)	9	7.2
Random Forrest	0.56 (0.4 – 0.72)	0.56 (0.45 – 0.67)	0.57 (0.45 – 0.69)	9	1.81

The Receiver Operator Curve plots for these 3 sets of results can be found in figure 5.3.



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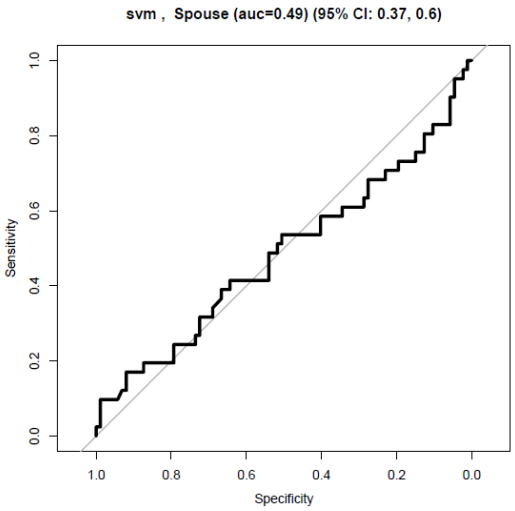
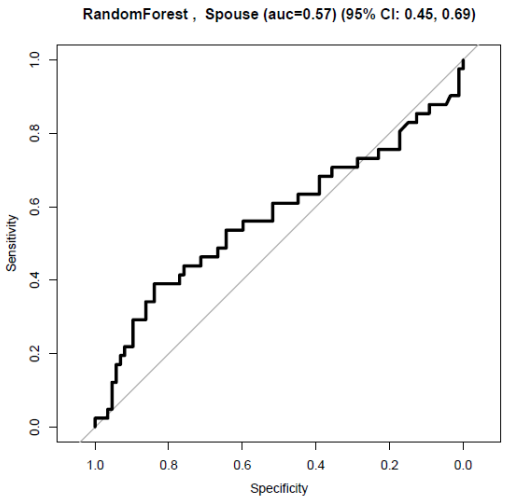


B.



C.

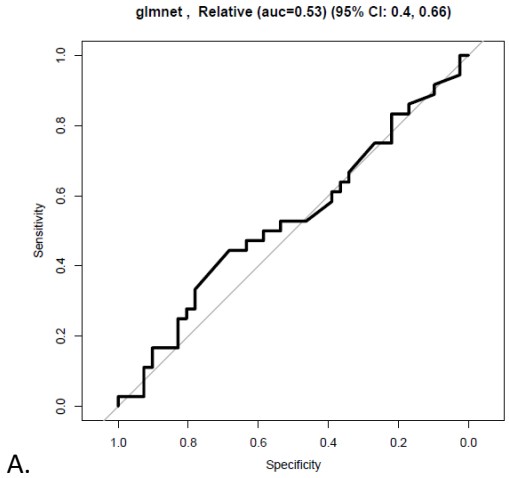
Figure 5.3. Receiver Operator Curve plots for statistical pipelines used to distinguish spouses from the pre-treatment CRC patients and relative groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

A comparison of the relative and spouse groups was made to determine whether the two control groups could be distinguished from each other.

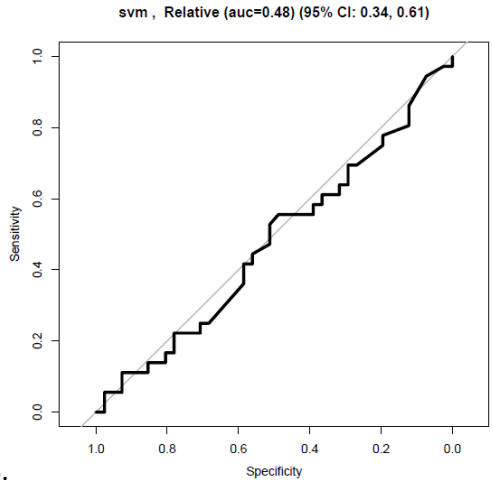
The results of the cross-validation were non-statistically significant and are as follows:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.53 (0.35 – 0.7)	0.54 (0.37 – 0.69)	0.53 (0.4 – 0.66)	6	3.79
Support vector Machine	0.5 (0.33 – 0.67)	0.51 (0.35 – 0.67)	0.48 (0.34 – 0.61)	6	4.3
Random Forrest	0.5 (0.33 – 0.67)	0.51 (0.35 – 0.67)	0.53 (0.4 – 0.66)	6	3.94

The Receiver Operator Curve plots for these 3 analyses can be found in figure 5.4.



A.



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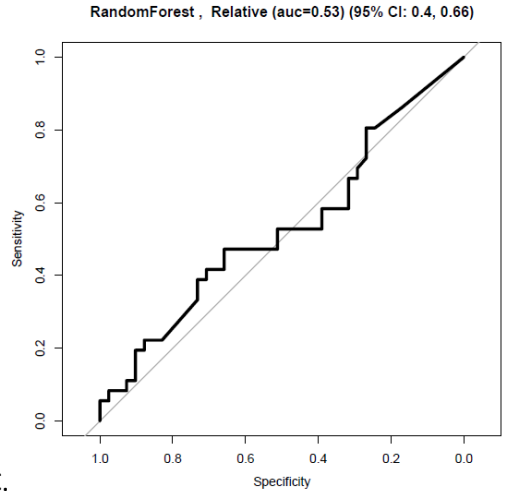


C.

Figure 5.4. Receiver Operator Curve plots for statistical pipelines used to distinguish the spouse/co-habitor group from the relative group. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

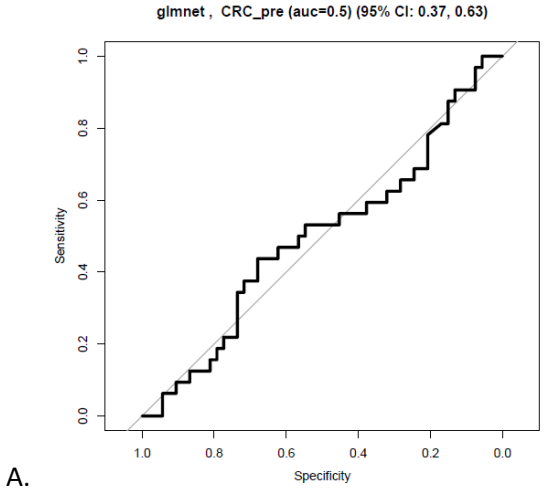
To further analyse the ability to distinguish the VOC profile of the pre-treatment CRC subjects, a subset analysis was performed. This time a smaller subset of CRC patients was used (n=35), and, to be included the CRC patients must have either a relative or a spouse sample included. This reduced the sample size for the CRC group, but not the relative or spouse group.

This analysis showed:

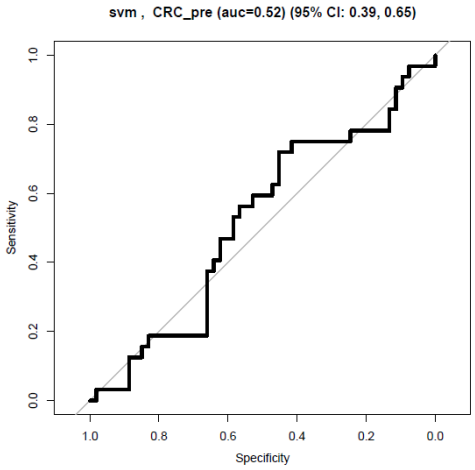
CRC patients

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.53 (0.35 – 0.71)	0.55 (0.4 – 0.68)	0.5 (0.37 – 0.63)	9	8.77
Support vector Machine	0.56 (0.38 – 0.74)	0.57 (0.42 – 0.7)	0.52 (0.39 – 0.65)	9	7.04
Random Forrest	0.56 (0.38 – 0.74)	0.57 (0.42 – 0.7)	0.55 (0.42 – 0.67)	9	4.39

The Receiver Operator Curve plots for these 3 analyses can be found in figure 5.5.



A.



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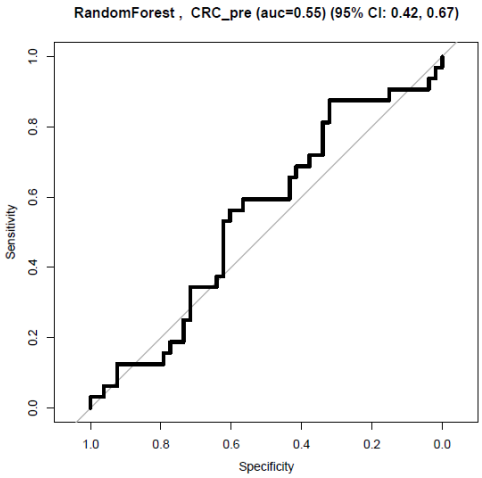


C.

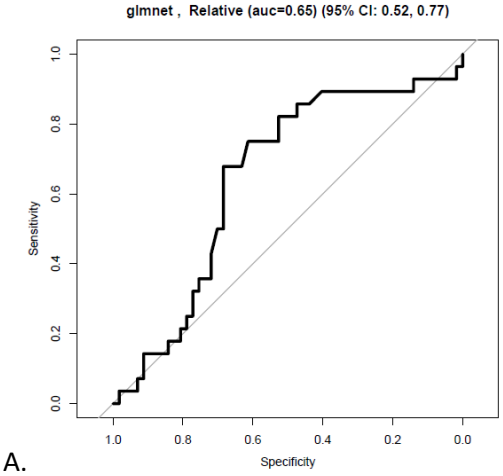
Figure 5.5. Receiver Operator Curve plots for statistical pipelines used to distinguish the reduced pre-treatment CRC patients from the relative and spouse/co-habitor control groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

A further cross validation, using this reduced number of CRC patients, was performed, with the relative and spouse groups each cross validated against all other groups e.g. relatives vs CRC and spouses, spouses vs CRC and relatives, and the following non statistically significant results were achieved:

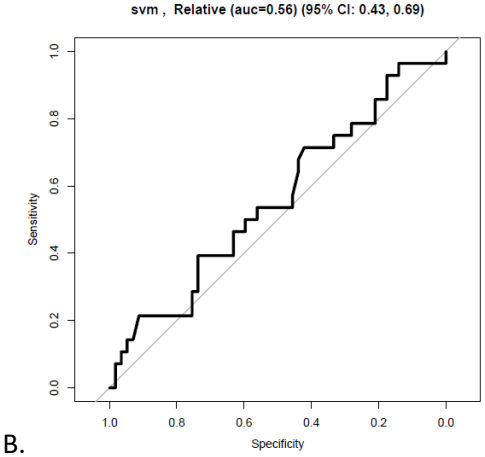
Relatives

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.68 (0.48 – 0.84)	0.68 (0.55 – 0.8)	0.65 (0.52 – 0.77)	9	0.261
Support vector Machine	0.54 (0.34 – 0.72)	0.54 (0.41 – 0.68)	0.56 (0.43 – 0.69)	9	3.55
Random Forrest	0.54 (0.34 – 0.72)	0.54 (0.41 – 0.68)	0.51 (0.38 – 0.64)	9	8.16

The Receiver Operator Curve plots for these 3 analyses can be found in figure 5.6.



A.



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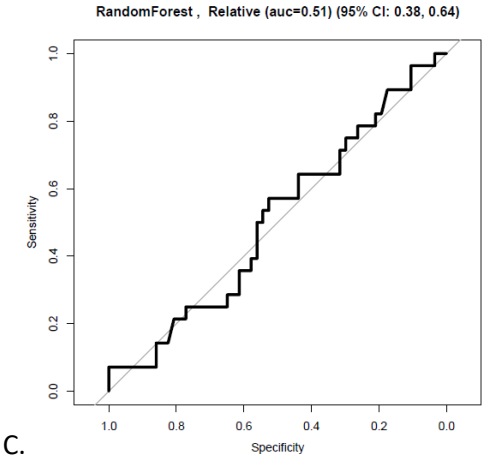


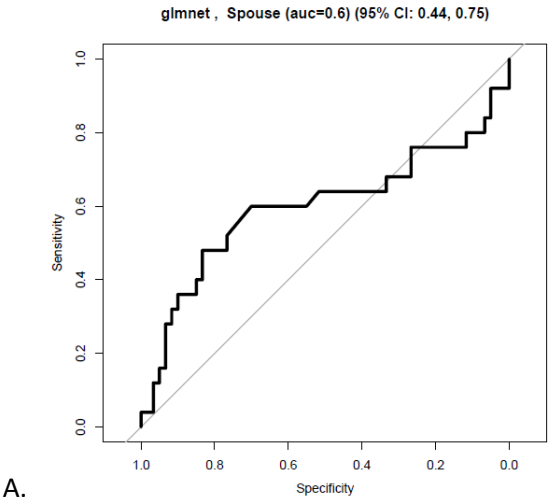
C.

Figure 5.6. Receiver Operator Curve plots for statistical pipelines used to distinguish the relative group from the reduced pre-treatment CRC and spouse/co-habitor groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

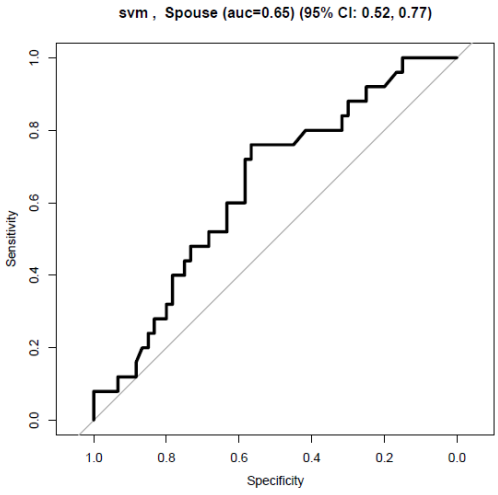
Spouses

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.6 (0.39 – 0.79)	0.6 (0.47 – 0.72)	0.6 (0.44 – 0.75)	9	1.44
Support vector Machine	0.6 (0.39 – 0.79)	0.6 (0.47 – 0.72)	0.65 (0.52 – 0.77)	9	0.327
Random Forrest	0.6 (0.39 – 0.79)	0.6 (0.47 – 0.72)	0.64 (0.52 – 0.76)	9	0.39

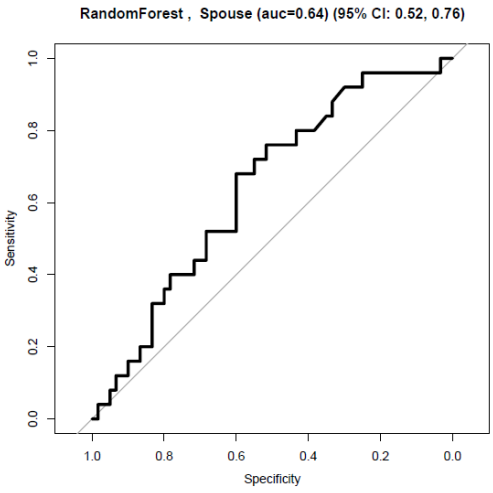
The Receiver Operator Curve plots for these 3 sets of results can be found in figure 5.7.



A.



B.



C.

Figure 5.7. Receiver Operator Curve plots for statistical pipelines used to distinguish the spouse/co-habitor group from the reduced pre-treatment CRC and relative groups. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

5.4. Discussion

The urinary VOC profiles of pre-treatment CRC patients were analysed using LC-FAIMS-MS technology and compared to the profiles of a first degree relative and a spouse/co-habitor. Statistical analysis utilised three different multi-class statistical classifiers in a 5-fold cross validation and demonstrated a significant difference between the CRC patients and their relatives and spouses for 2 of the models; sparse logistics regression and Random Forrest. The AUC for the sparse logistics regression model was 0.72, with a sensitivity of 0.63 and specificity of 0.64. The Bonferroni corrected p value was 0.000256. The Random Forrest classifier gave AUC, sensitivity and specificity of 0.71, 0.69 and 0.69 respectively, p = 0.00058. These results confirm that there is a distinct pattern of VOCs found in the urine of patients with CRC when compared to disease free controls. This confirms the findings of previous studies of urinary VOCs in CRC patients (115, 161-163), although this is the first study to do so using this precise FAIMS apparatus, the LC-FAIMS-MS.

The PPV was 54% for the sparse logistics regression model and 60% for the Random Forrest, whilst NPVs were 46% and 76% respectively. Whilst the positive predictive values compare very favourably to those demonstrated for FOBT and FIT of ~8-10% (85-87, 244) (Kearns, 85-87), the specificities and NPV compare less favourably to the 98% from previous studies (Kearns). The relatively poor specifities and NPV results represent one of the bigger obstacles that would need to be overcome for VOCs to enter clinical use as a screening tool for CRC.

There was no statistically significant difference in the urinary VOC profiles of the relatives and spouse cohorts when compared to each other. This suggests that the composition of the VOC profile in non-diseased individuals is very heterogenous, and that neither environment nor genetic factors cause a significant difference to the VOC profile, but that the presence of disease does. The reclassification analysis demonstrated that when the CRC cohort was combined with either the relative or spouse cohort, and then compared to the remaining cohort, the statistically significant difference was lost, with AUC, sensitivity and specificity falling to 0.49 - 0.63, 0.51 - 0.58 and 0.52 - 0.59 respectively across the 3 classifiers used. The p values were all non-statistically significant, with Bonferroni correction leading to values of 0.13 - 7.2. This result indicates that the addition of either the relative or spouse VOC profiles, to the CRC patient profiles, led to a dilution of the unique signal found within the CRC cohort. This dilution was to such an extent that the clear difference between a pure CRC cohort and the combined control profiles was disguised. This would suggest that the LC-FAIMS-MS technology has the ability to distinguish “disease” from “non-disease”, but that either it is unable to distinguish the two types of controls, or that the two types of controls have indistinguishable VOC profiles. The similarity of AUC, sensitivity and specificity of the reclassification of the genetic and the environmental controls suggests that the study may have been underpowered to detect these changes. Future studies would need to have larger numbers in each cohort to determine whether the lack of difference seen here was a result of underpowering or whether neither control cohort possess a VOC profile which is more similar to the CRC profile than the other

A sub-analysis of the cohorts, where samples were only included if there was a relative or spouse matched to the CRC patient sample, i.e. lone CRC samples were excluded, was also performed. This led to reduction in in CRC samples from 56 to 35. This analysis returned non-significant results with AUC, sensitivity and specificity of 0.5 - 0.55, 0.55 - 0.57 and 0.53 - 0.57 respectively, with p values of 4.39 - 8.77. The loss of statistical significance seen here is most likely due to the reduction in the sample size of CRC cohort, and hence underpowering of the analysis.

The results of the urinary VOC degradation study conducted in chapter 4 do raise the possibility that the observed effects here may have been diminished, or indeed accentuated by differences in the times that urine samples were stored at room temperature. Particularly the relatives cohort, as more samples from this cohort were returned via the postal service than for the other 2 groups. Some CRC samples were provided on the day of surgery and were frozen with 30 minutes of production. Others were done the same morning, and some the night before. Some specimens were returned via hospital courier, which would add several hours of exposure to atmospheric temperature. At the extreme end of the experiment, some samples, usually relative samples, were returned via the postal service, which, although first class postage was used, could result in a 48-72 hour exposure to atmospheric temperature. There was, however, no statistically significant difference between groups in the duration of exposure of the urine samples to atmospheric temperature and pressure. Over 90% of samples were frozen when they had been at room temperature for >12 hours but less than 48

hours, corresponding with the plateau phase of the degradation plots found in Chapter 4. This means that whilst confounding as a result of differing lengths of time from collection to freezing cannot be excluded, if present, it should have negligible effect. This means that the results of the main experiment, detailed in the next chapter, are valid within the context of sample degradation. No samples were exposed to atmospheric temperature for greater than 72 hours.

This wide ranging spectrum of times for storage at atmospheric temperature means that some samples will have been further along the degradation curve than others. This could result in skewing of results due to overly degraded samples, however, the majority of samples were returned within the 12-48 hour plateau phase and the numbers of samples from the two extremes of the time spectrum were equally distributed this effect should be negated.

The current theory of VOC generation suggests that VOCs represent the results of interactions between cellular metabolic processes, consumed food and medications and microbiome fermentation (96). The spouses/ co-habitors were all consuming the same food types as the CRC subjects and none of the 3 groups were consuming special diets (vegan, vegetarian, gluten free). This removes the potential for confounding in terms of food contributions to VOC profiles. There is the possibility that the relative and spouse groups were underpowered as discussed above, however no trends were noted and therefore it is unlikely that if present, and differences in VOC profiles between the control groups are unlikely to be significant.

There is the possibility that a different microbiome composition would produce different metabolic/ fermentation products from the same food substrate. This will be addressed in Chapter 7. The relatives would have a similar genetic constitution to the CRC patients and so a closer metabolic "fingerprint" than the spouses/ co-habitors. The fact that the CRC subjects are distinguishable from the relatives cohort, indicates that any differences in VOC profile would most likely be coming from either different food profile consumption, or a different metabolic profile as a result of the CRC, or different microbiome. This latter point will be addressed in Chapter 7. As the relatives and spouses had indistinguishable VOC profiles this suggests that neither food consumption nor microbiome can have significant influence over the VOC profile. Therefore, any differences observed must be a result of metabolic profile differences. As the relatives should share a closer metabolic profile to the CRC patients and yet are still distinguishable, this suggests that any difference in VOC profile should indeed be arising as a result of the presence of CRC, which could produces either a different profile of metabolites or cancer specific metabolites, detectable via the LC-FAIMS-MS technology.

This experiment demonstrates that CRC patients appear to have a unique urinary VOC profile, when compared to environmental and genetic controls. This adds to the growing body of evidence which indicates that urinary VOCs can be used to distinguish CRC patients from non-diseased controls, whether they are the independent controls used in previous studies, or the environmental or genetic controls used here. There was no statistical significance between the relative and spouse cohorts urinary VOC profiles, and when they were reclassified in

conjunction with the CRC patient samples, there did not appear to be a closer association between the either of the two control groups and the CRC subjects. This suggests that whilst environmental and genetic factors are believed to play a role in the development of sporadic CRC, that neither appears to result in a detectable VOC profile.

CHAPTER 6

Urinary VOC profiling of colorectal cancer patients prior to treatment and after treatment and by disease site and referral method

6.1 Introduction

As an extension of the primary study, the urinary VOC profiles of CRC patients were also studied in post-operative samples, which were collected at 3 and 6 month intervals after the subjects had undergone their initial surgery. This was to attempt to determine whether the VOC profile alters after the primary cancer has been removed.

At present the surveillance of post-treatment CRC patients includes a combination of colonoscopy, CT scanning and CEA blood monitoring. The aim of this experiment was to determine whether the VOC profiles of CRC patients could potentially be used as an alternative surveillance tool for detecting disease recurrence.

Further analysis was conducted to determine whether site of CRC (right sided, left sided, or rectal) affected VOC profile, and whether there was any difference with regards to referral method (2 week wait pathway, screening or other, which included emergency presentations, incidental findings and routine referrals).

6.2 Methods

CRC patients, who were recruited for the primary experiment, as described in section 3.2.1, were approached 3 and 6 months after their initial treatment. If they agreed to provide further samples then samples were collected and stored as described in sections 3.2.3. A further cohort of patients had samples collected. These were patients who have previously had curative resection of CRC, 24-36 months previously with no recurrence at the time of recruitment. This second cohort would act as a long term comparison group for the pre-treatment CRC patient profiles, the aim being to detect any changes that occur after patients have been cancer free for a prolonged period of 2 or more years.

The samples were frozen at -80ºC as soon as possible after their receipt. They were transferred to Owlstone, Cambridge in dry ice and, again, stored frozen at -80ºC. Samples were thawed and analysed using the LC-FAIMS-MS machine (Owlstone, UK), as described in section 3.3.1.

Data obtained from the LC-FAIMS-MS hybrid was pre-processed and analysed as described in sections 3.3.1.2a, and then underwent analysis using the statistical methods described in section 3.3.1.2b.

6.3. Results

As for the experiment outlined in Chapter 5, 56 CRC patients returned pre-operative samples. Of these, 23 returned samples after 3 months and 9 returned samples after 6 months. 30 CRC patients who were 24-36 months post initial treatment and currently in disease free surveillance also provided urine samples for VOC analysis.

The mean age of the pre-treatment group was 65.4 years (SD 11.5). For the 3 month post-treatment samples it was 66.5 (SD 13.0), and 64.7 (SD 12.7) for the 6 month post-treatment samples, and for the long term CRC follow up patients it was 66.5 (SD 11.7). There was no statistically significant difference between the ages of the groups using ANOVA analysis, $p = 0.57$.

There were 33 males and 23 females with CRC in the pre-treatment cohort; with 12 males and 11 females in the 3 month post-treatment cohort, 5 males and 4 females in the 6 month post treatment cohort and, finally, 20 males and 10 females in the long term CRC follow up cohort. The male: female distribution was analysed using the Chi squared test, and found to not be significantly different; chi squared statistic 1.2, $p = 0.75$.

The average cigarette consumption per day was 1.5 (SD 4.2) for the pre-treatment CRC group, 0.5 (SD 2.1) for the 3 month post-treatment cohort, 0 (SD 0) for the 6 month post-treatment cohort and 0.2 (SD 0.9) for the long term CRC follow up cohort. There was no statistically significant difference using ANOVA analysis, $p = 0.16$.

Average alcohol consumption (units per week) was 8.1 (SD 11.6) for the pre-treatment CRC group, 6.5 (SD 11.2) for the 3 month post-treatment cohort, 9.9 (SD 15.6) for the 6 month post-treatment cohort and 6.6 (SD 9.0) for the long term CRC follow up group. There was no statistically significant difference using ANOVA testing, $p = 0.83$.

No CRC subject had received recent courses of antibiotics prior to providing either pre-treatment or post-treatment samples. They had not undergone recent endoscopic investigation, so had not consumed bowel preparation medications.

Mean BMI for the CRC cohort was 27.5 (SD 5.2), 26.5 (SD 7.7) for the 3 month post-treatment cohort, 26.7 (SD 11.6) for the 6 month post-treatment cohort and 27.2 (SD 4.5) for the long term CRC follow up group. There was no statistically significant difference using ANOVA testing, $p = 0.93$.

The Duke's stages of the various groups are shown in table 6.4.1. There was no statistically significant difference seen in the grouping using Chi squared analysis; chi squared statistic 3.6, $p = 0.93$.

Duke's Stage	Pre-treatment CRC	3/12 CRC	6/12 CRC	Long term CRC
A (%)	8 (14.2%)	4 (17.4%)	1 (11.1%)	7 (23.3%)
B (%)	17 (30.4%)	7 (30.4%)	2 (22.2%)	10 (33.3%)
C1 (%)	20 (35.7%)	9 (39.1%)	5 (55.5%)	9 (30%)
C2 (%)	9 (16.1%)	2 (8.7%)	0 (0%)	3 (10%)

Table 6.1. Duke's stages of the pre-treatment CRC group, the 3/12 and 6/12 samples of the same group, and the long term CRC follow up group.

The anatomical distribution of the various groups is shown in table 6.4.2. There was no statistically significant difference seen in the grouping using Chi squared analysis; chi squared statistic 5.2, $p = 0.52$.

Anatomical distribution	Pre-treatment CRC	3/12 CRC	6/12 CRC	Long term CRC
Right sided (%)	24 (42.8%)	13 (56.5%)	4 (44.4%)	14 (46.7%)
Left sided (%)	17 (30.4%)	7 (30.4%)	4 (44.4%)	6 (20%)
Rectal (%)	15 (26.8%)	3 (13.4%)	1 (11.1%)	10 (33.3%)

Table 6.2. Anatomical distribution of the pre-treatment CRC group, the 3/12 and 6/12 samples of the same group, and the long term CRC follow up group.

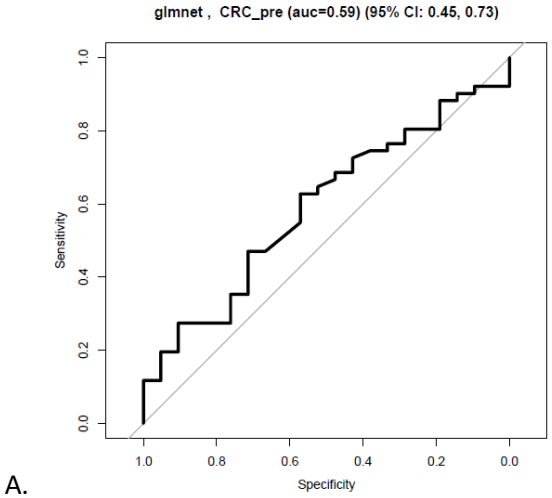
The referral pathways that the CRC patients were detected through were as follows: Screening programme = 18 (32.1%), 2 week wait pathway = 31 (55.4%), other (including routine, emergency and incidental findings) = 7 (12.5%). The long term CRC follow up patients were referred as follows: Screening programme = 5 (16.7%), 2 week wait pathway = 17 (56.7%), other (including routine, emergency and incidental findings) = 7 (23.3%). There was no statistically significant difference seen in the grouping using Chi squared analysis; chi squared statistic 3.2, $p = 0.20$.

The LC-FAIMS-MS data was analysed as described in section 5.3.2. The relevant subset of samples was extracted, and the different groups defined. 5-fold cross-validation was then used to assess classification accuracy across these groups, using three different multi-class classifiers: sparse logistic regression, Support Vector Machine, Random Forrest. This analysis generated outputs of one-vs-all ROC curves i.e. comparing a single group vs all other group(s), for example pre-treatment CRC vs 3 month post-treatment sample or for multiple comparisons pre-treatment CRC vs all post-treatment CRC (3 month and 6 month) and historic; all post-treatment CRC vs pre-treatment CRC and long term CRC follow up; long term CRC follow up vs pre-treatment and post-treatment CRC. Other results generated included the Area-Under-Curve (AUC) statistic, sensitivity/specificity values, which were selected automatically to be maximally similar given the ROC curve, and a p-value comparing the result to that expected for random chance (AUC=0.5), using a Wilcoxon rank-sum test.

The comparison of the LC-FAIMS-MS analysis of pre-operative CRC samples with the 3 month post-treatment samples. There was no statistical difference at 3 months compared to the pre-treatment samples.

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.57 (0.42 – 0.71)	0.57 (0.34 – 0.78)	0.59 (0.45 – 0.73)	6	1.35
Support vector Machine	0.53 (0.38 – 0.67)	0.52 (0.3 – 0.74)	0.5 (0.34 – 0.66)	6	5.76
Random Forrest	0.63 (0.48 – 0.76)	0.62 (0.38 – 0.82)	0.65 (0.51 – 0.78)	6	0.329

The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.1.



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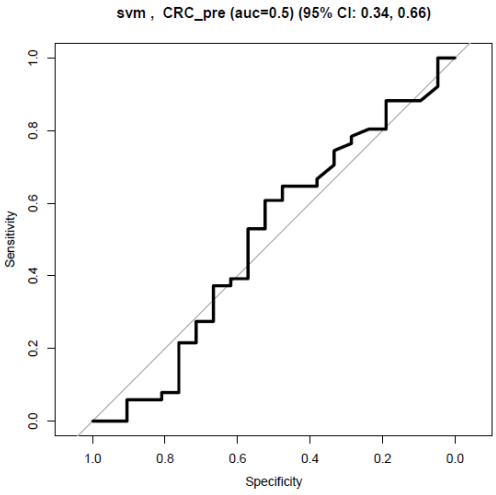

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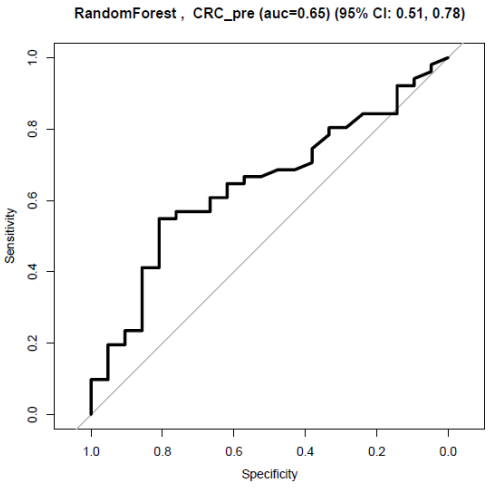


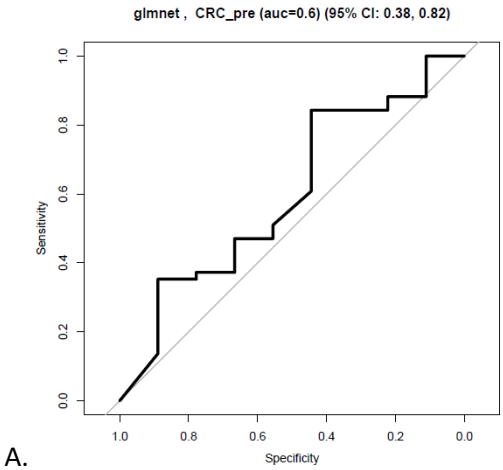
C.

Figure 6.1. Receiver Operator Curve plots for statistical pipelines used to distinguish pre-treatment CRC patients from the 3 month post-treatment group. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

The comparison of the LC-FAIMS-MS analysis of pre-operative CRC samples with the 6 month post-treatment samples. There was no statistical difference between the 6 month samples and the pre-treatment cohort.

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.51 (0.37 – 0.65)	0.56 (0.21 – 0.86)	0.6 (0.38 – 0.82)	6	2.1
Support vector Machine	0.65 (0.5 – 0.78)	0.67 (0.3 – 0.93)	0.59 (0.38 – 0.81)	6	2.33
Random Forrest	0.61 (0.46 – 0.74)	0.56 (0.21 – 0.86)	0.62 (0.42 – 0.82)	6	1.44

The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.2.



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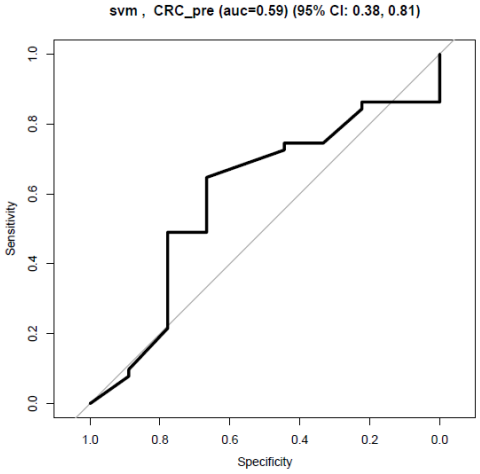

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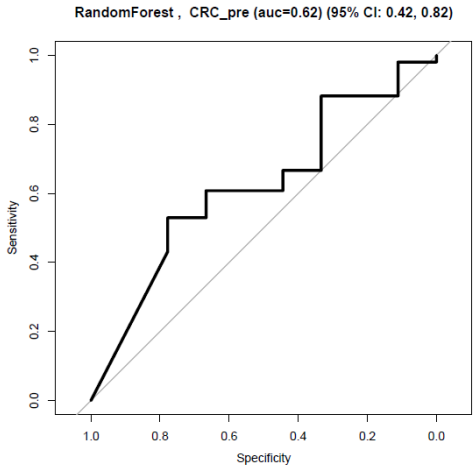


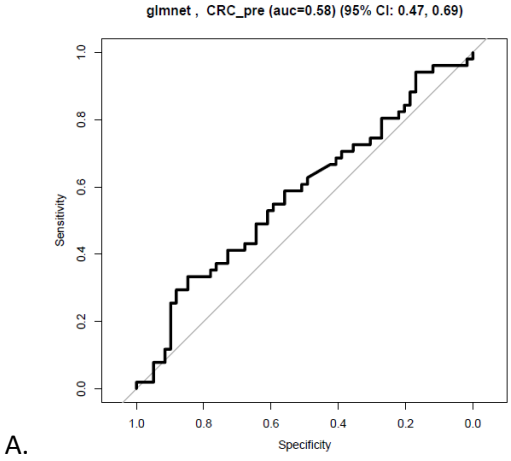
C.

Figure 6.2. Receiver Operator Curve plots for statistical pipelines used to distinguish pre-treatment CRC patients from the 6 month post-treatment group. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

An analysis was performed to see if pre-treatment CRC samples could be distinguished from grouped post-treatment 3 and 6 months samples, and also from long term CRC follow up patients. There was no statistical difference between the 3 patients cohorts.

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.57 (0.42 – 0.71)	0.56 (0.42 – 0.69)	0.58 (0.47 – 0.69)	9	1.34
Support vector Machine	0.51 (0.37 – 0.65)	0.51 (0.37 – 0.64)	0.52 (0.4 – 0.63)	9	7.04
Random Forrest	0.53 (0.38 – 0.67)	0.53 (0.39 – 0.66)	0.61 (0.5 – 0.71)	9	0.492

The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.3.



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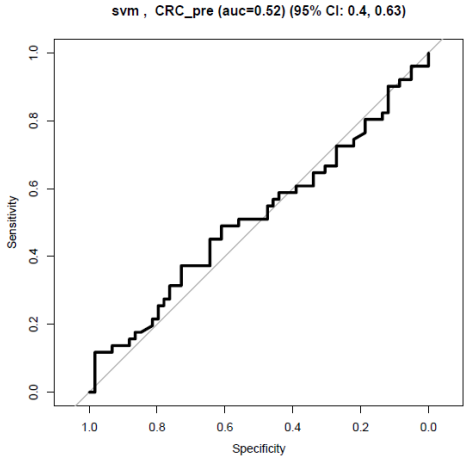

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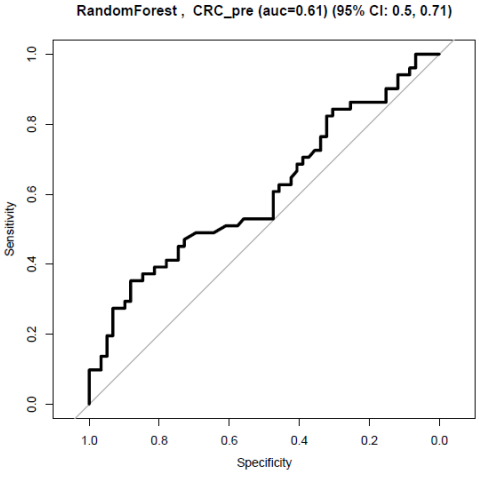


C.

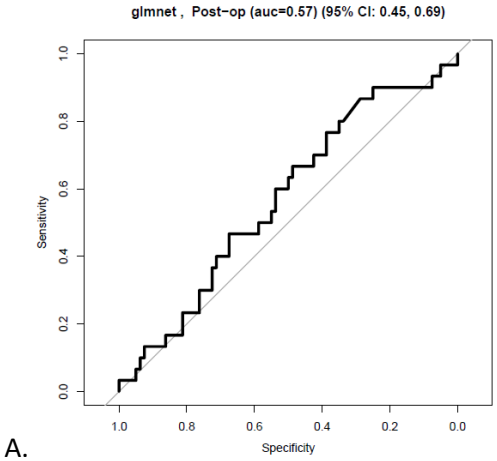
Figure 6.3. Receiver Operator Curve plots for statistical pipelines used to distinguish pre-treatment CRC patients from combined 3 and 6 month post-treatment groups and long term CRC follow up patients. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

When the post-treatment and long term CRC follow up groups were individually cross validated against a combination of the other 2 groups e.g. post-treatment vs pre-treatment CRC and long term CRC follow up, long term CRC follow up vs pre-treatment CRC and post-treatment CRC, the following results were achieved:

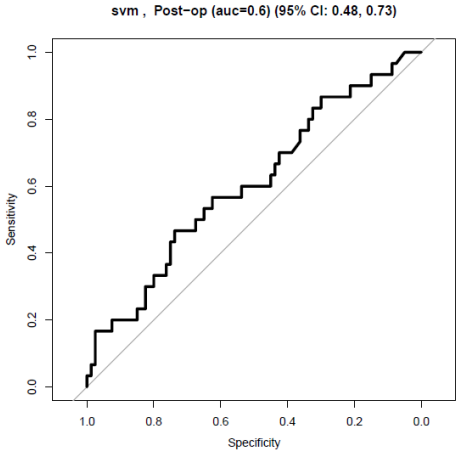
Post-treatment:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.53 (0.34 – 0.72)	0.54 (0.42 – 0.65)	0.57 (0.45 – 0.69)	9	2.37
Support vector Machine	0.57 (0.37 – 0.75)	0.56 (0.45 – 0.67)	0.6 (0.48 – 0.73)	9	0.823
Random Forrest	0.57 (0.37 – 0.75)	0.57 (0.46 – 0.68)	0.62 (0.5 – 0.73)	9	0.554

The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.4.



A.



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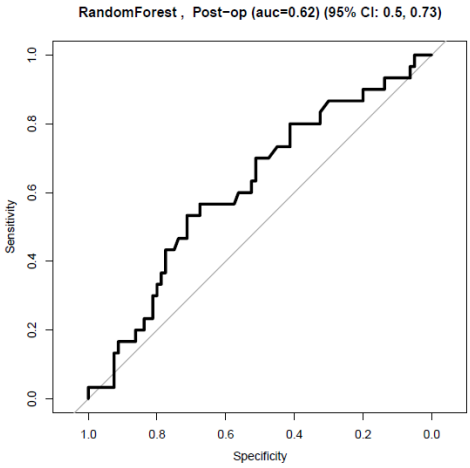


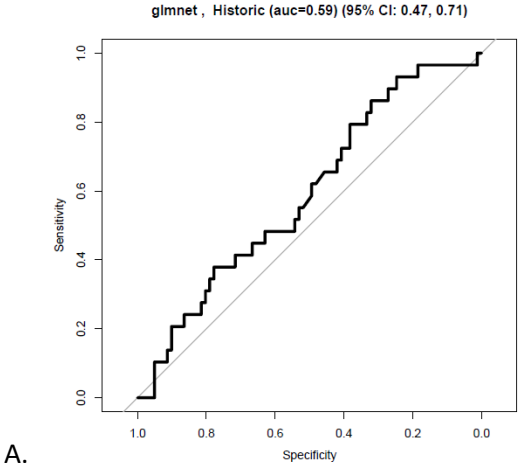
C.

Figure 6.4. Receiver Operator Curve plots for statistical pipelines used to distinguish combined 3 and 6 month post-treatment CRC patients from pre-treatment CRC and long term CRC follow up patients. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

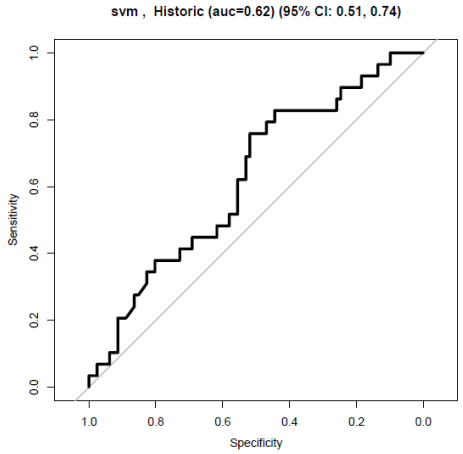
Long term CRC follow up:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.52 (0.33 – 0.71)	0.53 (0.42 – 0.64)	0.59 (0.47 – 0.71)	9	1.34
Support vector Machine	0.55 (0.36 – 0.74)	0.56 (0.44 – 0.67)	0.62 (0.51 – 0.74)	9	0.442
Random Forrest	0.66 (0.46 – 0.82)	0.64 (0.53 – 0.75)	0.65 (0.55 – 0.76)	9	0.129

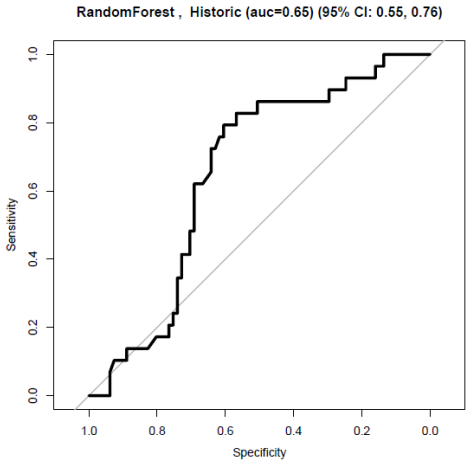
The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.5.



A.



B.



C.

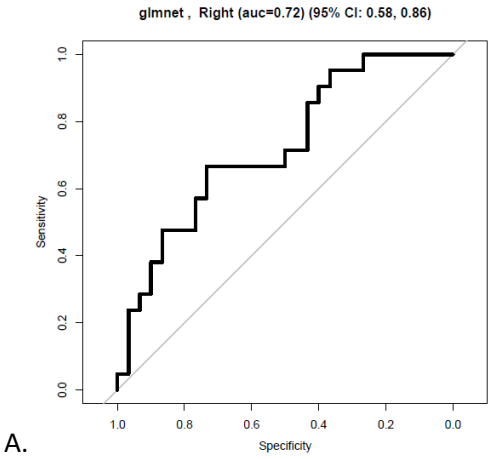
Figure 6.5. Receiver Operator Curve plots for statistical pipelines used to distinguish long term CRC follow up patients from combined 3 and 6 month post-treatment CRC patients and pre-treatment CRC. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

When the VOC data was analysed according to site of CRC, right, left or rectal, the analysis showed that sites could not be distinguished from each other with statistical significance:

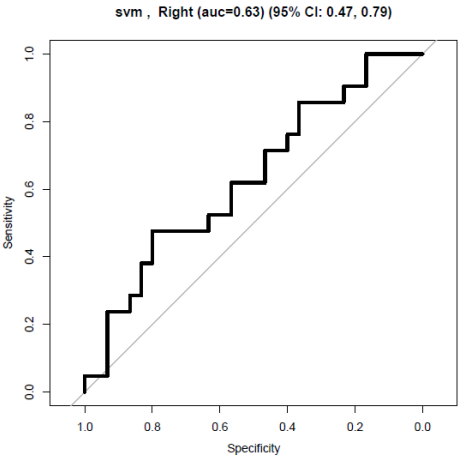
Right sided CRC:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.67 (0.43 – 0.85)	0.67 (0.47 – 0.83)	0.72 (0.58 – 0.86)	9	0.0683
Support vector Machine	0.57 (0.34 – 0.78)	0.57 (0.37 – 0.75)	0.63 (0.47 – 0.79)	9	1.03
Random Forrest	0.57 (0.34 – 0.78)	0.57 (0.37 – 0.75)	0.66 (0.5 – 0.82)	9	0.512

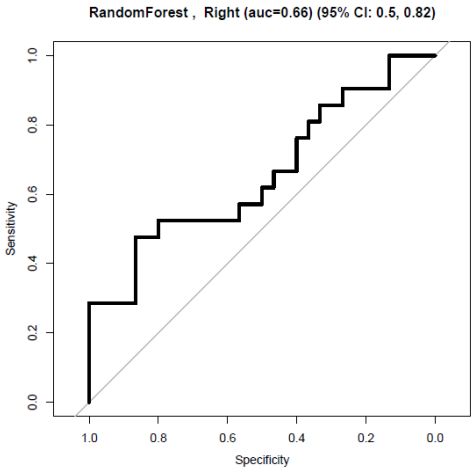
The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.6.



A.



B.



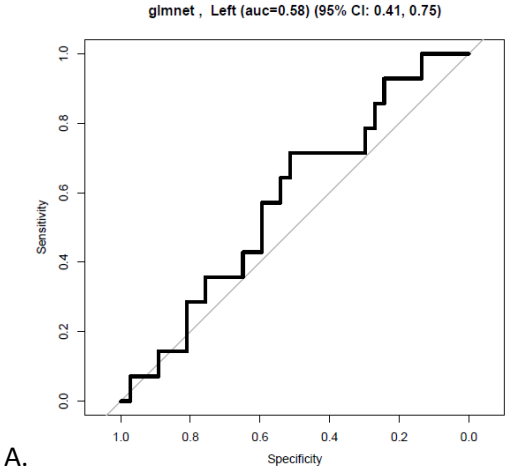
C.

Figure 6.6. Receiver Operator Curve plots for statistical pipelines used to distinguish right sided CRC from left sided and rectal CRC. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

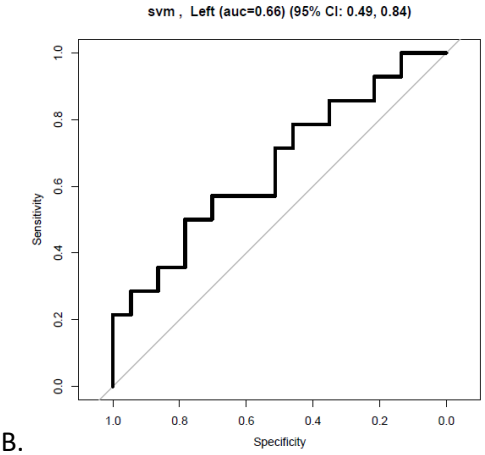
Left sided CRC:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.57 (0.29 – 0.82)	0.57 (0.39 – 0.73)	0.58 (0.41 – 0.75)	9	3.64
Support vector Machine	0.57 (0.29 – 0.82)	0.57 (0.39 – 0.73)	0.66 (0.49 – 0.84)	9	0.702
Random Forrest	0.57 (0.29 – 0.82)	0.57 (0.39 – 0.73)	0.66 (0.5 – 0.82)	9	0.67

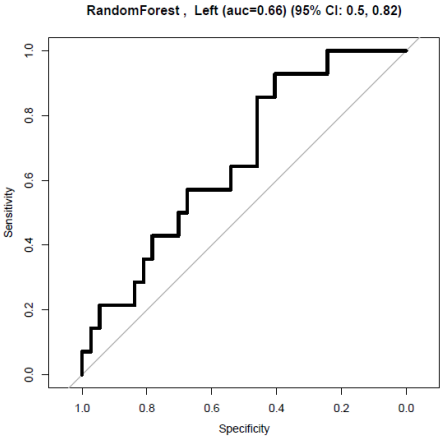
The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.7.



A.



B.



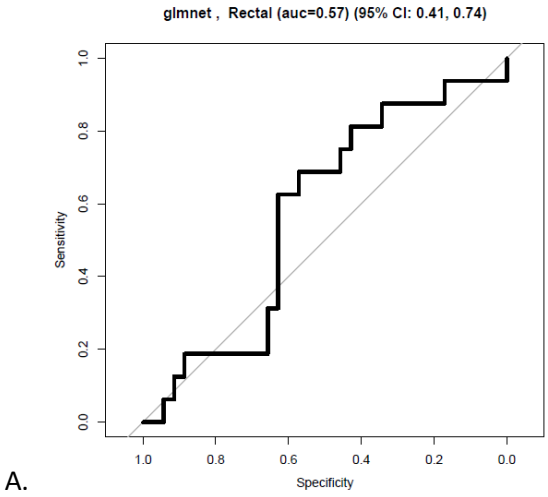
C.

Figure 6.7. Receiver Operator Curve plots for statistical pipelines used to distinguish left sided CRC from right sided and rectal CRC. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

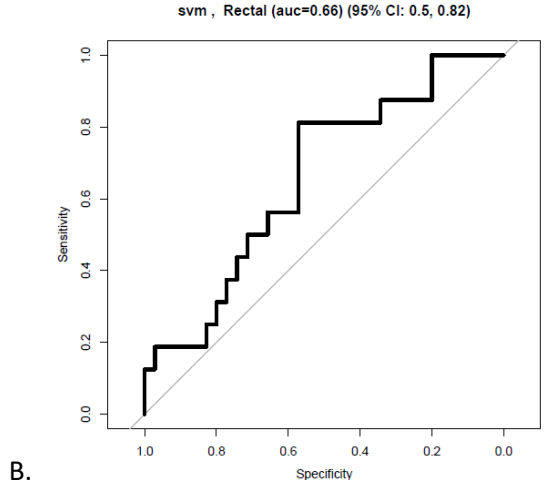
Rectal CRC:

Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.62 (0.35 – 0.85)	0.63 (0.45 – 0.79)	0.57 (0.41 – 0.74)	9	3.7
Support vector Machine	0.56 (0.3 – 0.8)	0.57 (0.39 – 0.74)	0.66 (0.5 – 0.82)	9	0.681
Random Forrest	0.62 (0.35 – 0.85)	0.6 (0.42 – 0.76)	0.65 (0.48 – 0.82)	9	0.846

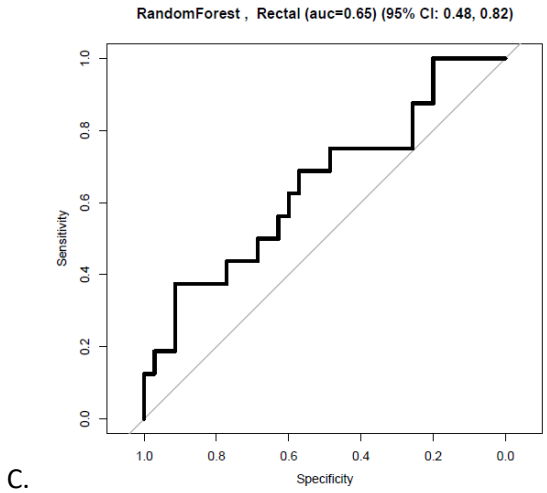
The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.8.



A.



B.



C.

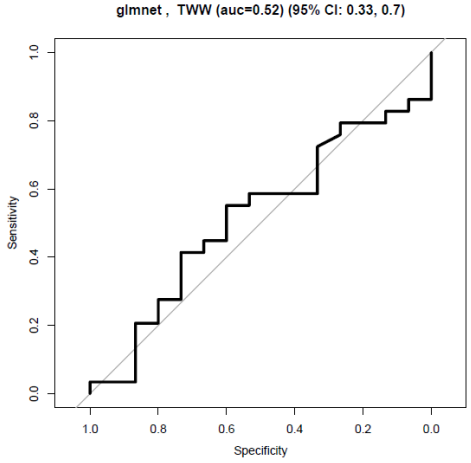
Figure 6.8. Receiver Operator Curve plots for statistical pipelines used to distinguish rectal CRC from left and right sided CRC. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

When the VOC data was analysed according to the method of referral for the CRC patients, screening programme or 2 week wait pathway, the analysis showed that sites could not be distinguished from each other with statistical significance. Patients referred by other pathways including incidental findings, routine referrals or emergency presentations were excluded due to small sample number (n=7).

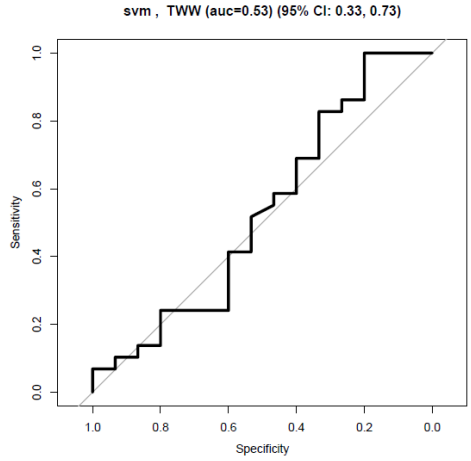
Statistical method	Sensitivity (Confidence intervals)	Specificity (Confidence intervals)	Area under Curve (Confidence intervals)	Number of hypotheses tested	Bonferroni corrected p value (*<0.05)
Sparse logistic regression	0.55 (0.36 – 0.74)	0.53 (0.27 – 0.79)	0.52 (0.33 – 0.7)	6	5.23
Support vector Machine	0.52 (0.33 – 0.71)	0.53 (0.27 – 0.79)	0.53 (0.33 – 0.73)	6	4.54
Random Forrest	0.52 (0.33 – 0.71)	0.53 (0.27 – 0.79)	0.53 (0.34 – 0.73)	6	4.32

The Receiver Operator Curve plots for these 3 analyses can be found in figures 6.9.

A.



B.



C.

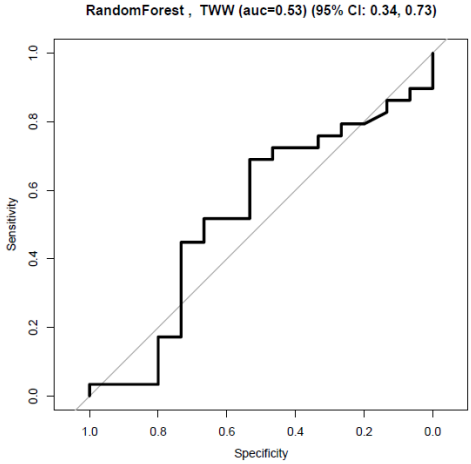


Figure 6.9. Receiver Operator Curve plots for statistical pipelines used to distinguish CRC patients referred through either 2 week wait pathway or through the screening programme. A. Sparse logistic regression B. Support vector machine C. Random Forest algorithm.

6.4 Discussion

As an extension of the experiment described in chapter 5, the urinary VOC profiles of CRC patients pre- and post-treatment were analysed using LC-FAIMS-MS technology. Three different multi-class statistical classifiers were utilised in a 5-fold cross validation, and demonstrated that there was no statistically significant difference between the pre-treatment CRC samples and any of the various post-treatment samples (3 and 6 months), nor when all post-treatment samples were combined. There was also no statistically significant difference between the pre-operative CRC patients samples and samples collected from long term CRC follow up subjects, who were 24-36 months post curative treatment and were in disease-free surveillance.

These findings suggest that there may be no role for the use of urinary VOCs in the surveillance for CRC recurrence, as those who have had the disease, but are now "cured", are indistinguishable from those who have active disease. The underlying reasons for this lack of change in VOC profile after CRC removal would need to be further explored. It could represent an underpowering of the study, or that any differences between the cohorts are very small. As discussed in chapter 5, the various contributing factors to VOC profile are believed to be metabolic processes, diet and medication, and microbiome profile. The microbiome of pre and post-treatment CRC patients will be addressed in chapter 8. No patients altered their diets between diagnosis of cancer and providing post-treatment samples. No

patients were started on any new medication, other than chemotherapy for some of the Duke's B and all of the Duke's C patients.

A sub-analysis of the post-treatment samples according to Duke's stage, and whether or not they had chemotherapy, was not undertaken due to small sample sizes (see table 6.1), with 14 out of 23 patients who returned samples at 3 months receiving chemotherapy and 7 out of 9 patients who returned samples at 6 months receiving chemotherapy. However, as the post-treatment samples could not be distinguished from pre-treatment, this indicates that again, either the experiment is underpowered or that chemotherapy does not appear to have had a significant effect on VOC profile. If it is the latter then this could suggest that any changes in metabolome of the CRC patients in response to CRC removal, have not led to a significant change in the VOC profile. Thus, indicating, that any metabolic changes which occur in the presence of CRC, are long term changes and persist even after the CRC has been treated.

It is possible that there is a difference in the urinary VOC profile of pre and post-treatment CRC samples; however, if present they may be so small to be of questionable clinical significance. It could be that the LC-FAIMS-MS machine is not sensitive enough, or not configured in the right way to detect these differences. It could also be that the statistical methods used are insufficiently fine-tuned to allow detection of these differences. Both the technology and the statistical methods are novel and have never been used in this precise form to distinguish patients with CRC from urinary VOCs before, and so, with further improvements

to the technology and the statistical methods, differentiation of pre and post-treatment CRC subjects may become possible.

There is no conclusive evidence that VOCs can be utilised in other cancers for disease recurrence. Two studies into the use of exhaled VOCs in lung cancer have shown variable results, with some alteration in VOC profile in post-treatment samples, but the VOC profile did not revert completely back to that of healthy controls (150, 212).

There has been promising evidence to date, of the value of the use of VOCs in surveillance for CRC recurrence. Altomare et al found that by using GC-MS to analyse exhaled breath of CRC patients, 2 years after curative surgery, they were able to distinguish pre-operative from post-treatment samples with a sensitivity of 100%, specificity of 97%, accuracy of 98.8% and AUC 1 (211). Ma et al had previously demonstrated that there is a significant reduction in the levels of 2 individual VOCs post operatively in urinary VOCs, using GC-MS analysis, although did not recommend their use in surveying for disease recurrence (161).

There were also no statistically significant differences in the urinary VOC profiles of CRC patients with different sites of CRC (right, left and rectal) or by method of referral (2 week wait, screening) This indicates that the CRC itself produces a consistently unique VOC profile, which is not affected by differing site of cancer or method of discovery. To date, there have been no studies into the VOC profiles of CRC from different sites or referral pathways. This would suggest that urinary VOC analysis could be a viable method for detecting all types of CRC from all referral

pathways, as the VOC changes detected are found in all subtypes of adenocarcinoma CRC.

The results of this experiment suggest that whilst urinary VOC analysis has the potential to be used to distinguish patients with CRC compared to controls, as described in Chapter 5, the value of VOC analysis in monitoring for disease recurrence is limited at this stage, as the unique VOC signature found in pre-treatment CRC patients persists after they have had treatment, and is indistinguishable from patients who are in disease free remission 24-36 months later. Further work is needed in this area, and larger studies required, with longer term follow up to determine whether VOC analysis could be a useful tool in CRC surveillance, or, if its future lies in disease detection only.

CHAPTER 7

Stool 16s RNA profiling of colorectal cancer patients, their first degree relatives and co-habitors

7.1. Introduction

16s RNA profiling of the microbiome of pre-treatment colorectal cancer patients, their first degree relatives and spouses/co-habitors was undertaken as part of the experiments of this thesis.

The aim was to determine whether CRC patients have a unique microbiome profile, which is distinct from those who share genetic traits, and those who share an environment. Previous studies into microbiome profiling of CRC patients have shown a restricted bacterial profile compared to healthy controls, termed dysbiosis. However, these experiments will use environmental and genetic controls to determine whether the CRC patients profile is indeed distinct and, if so, does it more closely resemble that of those individuals who share genetic material with the CRC patients, or, those who share an environment.

This experiment was planned to allow greater interpretation of the VOC data described in chapter 5, and to help to provide information as to whether VOC profiles could be affected by different microbiome composition.

7.2. Methods

CRC patients were recruited as described in section 3.2.1. Relatives and spouses of the CRC patients were recruited as described in section 3.2.2. Only first degree relatives were approached to preserve as much of the genetic "signal" as possible. This included siblings and children. Children still co-habiting with the CRC patients were excluded as they would represent both genetic and environmental controls and could lead to confounding. Sample collection and storage was performed as described in section 3.2.4. The samples were frozen at -80^{o}C as soon as possible after their receipt. They were transferred to the University of Warwick in dry ice and, again, stored frozen at -80^{o}C. Samples were thawed and analysed using 16s RNA sequencing on an Illumina MiSeq platform as described in section 3.3.3. Processing of the sequence data and analysis was performed as described in section 3.3.3.7.

7.3. Results

As described in Chapter 5, 72 CRC patients were recruited, in addition to 61 first degree relatives and 56 spouses. Stool samples were returned by 44 pre-treatment CRC patients, 34 first degree relatives and 39 spouses.

The mean ages of the three cohorts were 65.3 years (SD 11.2), 51.2 years (SD 12.6) and 59.9 years (SD 12.4) respectively. Normal distribution of ages was confirmed using Shapiro-Wilk testing. Analysis of Variance (ANOVA) testing and post-hoc Tukey's honest significance difference (HSD) test showed no statistically significant differences in ages between CRC patients and their spouses. However, there was a significant difference between both CRC and first degree relatives, and spouses and first degree relatives, $p < 0.01$. This is due to presence of both siblings and children of the cancer patients in the sample mix. This is an unavoidable consequence of recruiting children of the cancer patients.

There were 25 males and 19 females with CRC in the final cohort, this ratio, was expectedly, reversed in the spouse cohort, with 13 males and 26 females. The male to female ratio in the relative cohort was 14 to 18. The male: female distribution was analysed using the Chi squared test, and found to not be significantly different, chi squared statistic 4.7, $p = 0.1$.

The average cigarette consumption per day was 0.73 (SD 2.6) for CRC patients, 2.9 (SD 5.6) for first degree relatives, and 2 (SD 6.4) for spouses. There was no statistically significant difference using ANOVA testing, $p = 0.24$.

Average alcohol consumption (units per week) was 7.8 (SD 11.6) for CRC patients, 6.8 (SD 7.0) for first degree relatives and 8.1 (SD 10.9) for spouses. There was no statistically significant difference using ANOVA testing, p = 0.94.

Mean BMI for the CRC cohort was 27.4 (SD 4.3), 25.3 (SD 4.1) for the first degree relatives and 26.6 (SD 4.8) for the spouses. There was no statistically significant difference using ANOVA testing, p = 0.30.

The Duke's stage of the CRC patients was as follows: A = 8 (18.2%), B = 15 (34.1%), C1 =13 (29.5%), C2 =5 (11.4%). The anatomical distribution of the CRC subjects was: right sided = 18 (40.1%); left sided = 14 (31.2%) and rectal = 12 (27.3%). The referral pathways that the CRC patients were detected through were: Screening programme = 17 (40.5%), 2 week wait pathway = 21 (50%), and other (including routine, emergency and incidental findings) =6 (9.5%).

The demographics of the recruited subjects can be found in table 7.1.

No CRC patients, relatives or spouses had received recent courses of antibiotics. The CRC subjects had not undergone colonoscopy, and hence consumed bowel preparation medication, within the previous 2 weeks.

3 CRC patients had a first degree relative (sibling or parent) with a history of CRC, these relatives were all diagnosed over the age of 60, meaning that all of our patients were sporadic CRC patients.

Group	Pre-treatment CRC	Relative	Spouse	P value
Samples	44	34	39	
Mean age (SD)	65.3 (SD 11.2)	51.2 (SD 12.6)*	59.9 (SD 12.4)	*<0.01
Sex (M:F)	25:19	15:19	13:26	0.1
Number of cigarettes smoked per day (SD)	0.73 (2.6)	2.9 (5.6)	2 (6.4)	0.24
Alcohol units per week (SD)	7.8 (11.6)	6.8 (7.0)	8.1 (10.9)	0.94
Mean BMI (SD)	27.4 (4.3)	25.3 (4.1)	26.6 (4.8)	0.30
Dukes stage (%)				
A	9 (18.2%)	-	-	
B	15 (34.1%)	-	-	
C1	13 (29.5%)	-	-	
C2	5 (11.4%)	-	-	
Site (%)				
Right	18 (40.1%)	-	-	
Left	14 (31.2%)	-	-	
Rectal	12 (27.3%)	-	-	
Referral Route (%)				
Screening	17 (40.5%)	-	-	
2WW	21 (50%)	-	-	
Other	6 (9.5%)	-	-	

Table 7.1 Demographic data from recruited pre-treatment CRC patients, their first degree relatives and co-habitors/spouses.

The 16s microbiome OTU data was analysed as described in section 3.3.3.7. Once the raw sequence data had been merged, quality controlled and filtered to exclude low quality reads, with counts of less than 5000 reads, 10 samples out of 179 had been excluded.

This left remaining sample sizes of 41 for pre-treatment CRC, 38 spouses and 33 relatives.

There were 1346 OTUs identified across all samples, including post-treatment samples. The data on these will be discussed in Chapter 8.

Relative abundance plots of the pre-treatment CRC samples and healthy controls were made. These can be found in figure 7.1.

An analysis of similarity (ANOSIM) test was run in QIIME using the script *compare_categories.py.* The ANOSIM script is a non-parametric test which determines whether two or more groups of samples are significantly different. The ANOSIM analysis returned an R value of 0.067 ($p=<0.001$). As the R value is close to 0, this indicates that the groups contain an even distribution of species and as such the pre-treatment CRC samples are very similar to the controls (relatives and spouses).

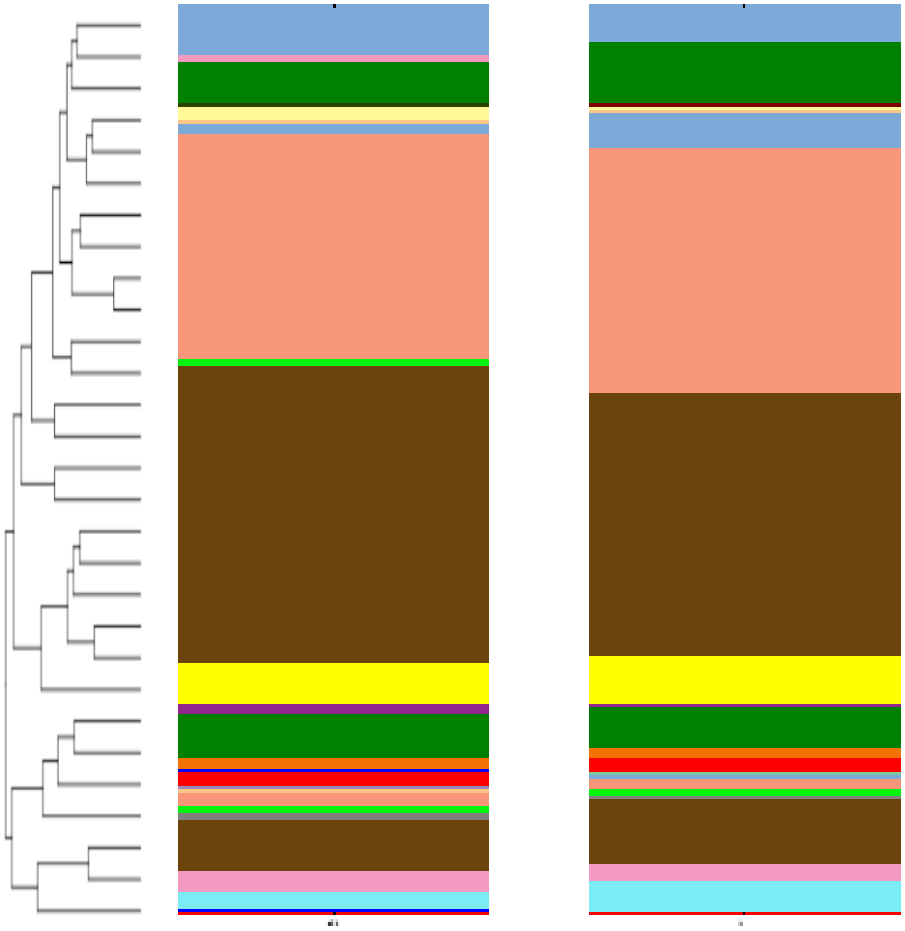


Figure 7.1. Relative abundance plots of pre-treatment CRC subjects (left) and all healthy controls: relatives and spouses (right).

The QIIME script *group significance.py* was used to compare the pre-treatment CRC samples with all controls. Within this the Kruskal-Wallis test was performed to check for significant differences between the groups. This script runs a non-parametric ANOVA test on OTU frequencies in sample groups to ascertain whether or not there are statistically significant differences between the OTU abundance in the different sample groups.

Of the 1346 OTUs identified there were 82 (6.2%) which were significantly different between the CRC subjects and all controls (relatives and spouses). These are shown in Table 7.2. Of characterised OTUs, 82 out 1346 (6.2%) were significantly different between the CRCs and controls, 46 were more common among CRC samples, with 31 of these (67%) corresponding to clostridales and 36 were more common among the controls, with 33 (92%) identified as clostridales. The most predominant clostridiales identified was ruminoccus sp. with 16/82 (19.5%) OTUs characterised as such.

To further analyse the microbiome differences a subset analysis was performed. This time a smaller subset of CRC patients was used (n=31), and, to be included the CRC patients must have either a relative or a spouse sample included. This reduced the sample sizes to 31 for the CRC group, 27 for the relative and 23 for spouses. This analysis determined that there were 45 OTUs (3.3%) with statistically significant expression levels between the CRC and controls. These are shown in Table 7.3. Of the 45 identified OTUs, 12 (27%) had higher expression levels in CRC subjects, with 9 identified as clostridiales.

Comparison of the larger analysis and the subset analysis revealed a subset of 22 OTUs which were found to have significantly different levels of expression in both analyses. These can be found in Table 7.4 along with their corresponding order/species. Of the 22 OTUs, 8 were found to have higher abundance levels in CRC subjects (6 Clostridiales) and 14 lower abundance levels than the control groups (12 Clostridiales).

Principal component analysis of the overall diversity of the 2 main groups showed no statistically significant differences between the microbiome composition of the pre-treatment CRC subjects and the healthy controls. See figure 7.2.

Comparison of the healthy control groups (relatives vs spouses) using Kruskal-Wallis testing identified 567 OTUs, with 25 (4.4%), showing statistically significant differences in relative abundance between the two control groups (14 with increased abundance in relatives and 11 in spouses). Of these different OTUs, 18 (72%) were clostridiales. These are shown in Table 7.5.

OTU	Test-Statistic	Pre-treatment mean	Healthy controls mean	Taxonomy order/family	species	P
OTU_95	42.15	47.93	0.07	Clostridiales	Peptostreptococcus anaerobius	0.000
OTU_220	24.49	7.38	0.17	Clostridiales	Parvimonas sp	0.000
OTU_760	21.11	2.81	0.03	Fusobacteriales	Fusobacterium sp	0.000
OTU_197	12.17	23.48	0.20	Bacteroidales	Porphyromonas sp	0.000
OTU_674	11.31	0.86	0.15	Gemellales	-	0.001
OTU_213	10.74	0.74	4.27	Clostridiales		0.001
OTU_519	10.61	1.26	0.00	Clostridiales	Dialister sp	0.001
OTU_35	9.00	88.14	24.35	Clostridiales	Ruminococcus gnavus	0.003
OTU_236	8.77	0.26	1.83	Clostridiales	Ruminococcus sp	0.003
OTU_453	8.76	1.00	0.00	Erysipelotrichales	Bulleidia sp	0.003
OTU_335	8.76	0.26	0.00	Clostridiales	Anaerococcus sp	0.003
OTU_1053	8.71	1.48	0.90	Clostridiales	-	0.003
OTU_11	8.32	17.79	107.41	Clostridiales	Dialister sp	0.004
OTU_569	8.25	0.48	0.07	Clostridiales	Pseudoramibacter Eubacterium sp	0.004
OTU_606	8.14	3.07	26.75	Clostridiales	Dialister sp	0.004
OTU_448	8.06	1.24	0.21	Clostridiales	-	0.005
OTU_316	7.98	0.98	2.76	Clostridiales	Dialister sp	0.005
OTU_407	7.86	1.60	5.41	Clostridiales	Lachnospira sp	0.005
OTU_151	7.84	0.00	2.99	Clostridiales	Ruminococcus sp	0.005
OTU_38	7.26	5.74	12.85	Clostridiales	Lachnospira sp	0.007
OTU_970	7.13	0.00	0.20	Clostridiales	-	0.008
OTU_982	6.97	0.64	0.04	Clostridiales	Ruminococcus sp	0.008
OTU_628	6.95	0.10	0.00	Erysipelotrichales	-	0.008
OTU_429	6.95	1.38	0.00	Fusobacteriales	Fusobacterium sp	0.008
OTU_294	6.84	0.76	1.94	Burkholderiales	Sutterella sp	0.009
OTU_219	6.64	2.36	4.63	Clostridiales	-	0.010
OTU_126	6.56	4.86	12.08	Bacteroidales	Bacteroides sp	0.010
OTU_1027	6.43	0.00	0.14	Clostridiales	Ruminococcus sp	0.011
OTU_430	6.34	0.64	0.25	Clostridiales	Ruminococcus sp	0.012
OTU_939	6.30	1.50	0.61	Clostridiales	Ruminococcus sp	0.012
OTU_256	6.28	1.05	0.87	Clostridiales	Anaerotruncus sp	0.012
OTU_573	5.93	0.05	0.30	Clostridiales	-	0.015
OTU_1317	5.80	0.33	0.01	Bacteroidales	Odoribacter sp	0.016
OTU_215	5.79	2.60	0.23	Erysipelotrichales	Eubacterium cylindroides	0.016
OTU_31	5.77	20.29	0.38	Synergistales	-	0.016
OTU_481	5.77	0.14	0.01	Clostridiales	-	0.016
OTU_921	5.76	4.67	0.10	Lactobacillales	Streptococcus sp	0.016
OTU_560	5.50	0.02	0.27	Clostridiales	-	0.019

OTU_113	5.41	17.81	3.48	Clostridiales	Ruminococcus sp	0.020
OTU_264	5.29	1.76	1.18	Clostridiales	Dorea sp	0.021
OTU_465	5.25	0.64	0.08	Clostridiales	-	0.022
OTU_433	5.24	0.38	0.87	Clostridiales	Oscillospira sp	0.022
OTU_192	5.21	0.98	2.24	Clostridiales	Ruminococcus sp	0.022
OTU_238	5.19	30.14	35.48	Clostridiales	Ruminococcus sp	0.023
OTU_218	5.17	2.83	5.30	Clostridiales	-	0.023
OTU_525	5.16	1.17	0.00	Bacteroidales	-	0.023
OTU_450	5.16	0.31	0.00	Coriobacteriales	-	0.023
OTU_1225	5.16	0.17	0.00	Clostridiales	-	0.023
OTU_616	5.16	0.10	0.00	Clostridiales	Ruminococcus sp	0.023
OTU_444	5.16	1.19	0.00	Clostridiales	Ruminococcus sp	0.023
OTU_920	5.16	1.05	0.00	Clostridiales	Anaerococcus sp	0.023
OTU_648	5.04	0.00	0.24	Clostridiales	Veillonella dispar	0.025
OTU_416	4.93	0.05	0.24	Clostridiales	-	0.026
OTU_383	4.87	72.24	100.65	Clostridiales	Faecalibacterium prausnitzii	0.027
OTU_139	4.82	0.95	9.68	Clostridiales	-	0.028
OTU_83	4.70	1.55	7.15	Coriobacteriales	-	0.030
OTU_395	4.52	0.05	0.65	Clostridiales	Ruminococcus sp	0.033
OTU_348	4.48	0.05	0.59	Clostridiales	Ruminococcus sp	0.034
OTU_231	4.48	0.98	2.03	Clostridiales	Ruminococcus sp	0.034
OTU_835	4.40	0.60	0.30	Clostridiales	Ruminococcus sp	0.036
OTU_1278	4.37	0.00	0.34	Clostridiales	Ruminococcus sp	0.037
OTU_181	4.33	6.07	2.07	Turicibacterales	Turicibacter sp	0.037
OTU_1026	4.33	0.29	0.58	Clostridiales	-	0.037
OTU_244	4.31	2.88	5.03	Clostridiales	-	0.038
OTU_186	4.27	3.17	0.61	Coriobacteriales	-	0.039
OTU_589	4.25	0.21	0.61	Clostridiales	Anaerococcus sp	0.039
OTU_510	4.22	6.14	9.62	Clostridiales	Faecalibacterium prausnitzii	0.040
OTU_487	4.20	4.38	2.28	Clostridiales	-	0.040
OTU_132	4.17	4.10	1.76	Clostridiales	-	0.041
OTU_404	4.14	3.12	0.01	Bacteroidales	Porphyromonas sp	0.042
OTU_985	4.07	0.10	0.01	Clostridiales	-	0.044
OTU_771	4.05	0.24	0.56	Clostridiales	-	0.044
OTU_895	4.04	0.36	0.03	Clostridiales	-	0.045
OTU_1269	4.02	0.07	0.32	Clostridiales	-	0.045
OTU_301	4.02	11.90	3.55	Clostridiales	Dorea sp	0.045
OTU_308	3.99	1.79	1.58	Clostridiales	Ruminococcus gnavus	0.046
OTU_873	3.94	0.31	0.13	Clostridiales	-	0.047
OTU_908	3.93	0.50	0.21	Clostridiales	-	0.047
OTU_1044	3.91	0.05	0.21	Clostridiales	Ruminococcus sp	0.048

OTU_81	3.91	4.55	0.04	Clostridiales	Succiniclasticum sp	0.048
OTU_565	3.88	1.95	2.97	Clostridiales	Coprococcus sp	0.049
OTU_418	3.85	0.86	0.20	Clostridiales	Epulopiscium sp	0.049

Table 7.2. Table of OTUs which were statistically significant in distribution between the pre-treatment CRC samples and all controls (relatives and spouses). OTUs with higher abundance in CRC subjects are highlighted grey. Relative abundances and p values included

OTU	Test-Statistic	Pre-treatment CRC mean	Spouse mean	Relative mean	taxonoic order/family	species	P
OTU_95	31.52	42.62	0.05	0.08	Clostridiales	Peptostreptococcus anaerobius	<0.001
OTU_220	15.24	7.86	0.05	0.38	Clostridiales	Parvimonas sp	<0.001
OTU_760	13.43	3.07	0.09	0.00	Fusobacteriales	Fusobacterium sp	0.001
OTU_1224	13.10	4.55	2.68	9.42	Bacteroidales	Bacteroides sp	0.001
OTU_213	12.45	0.48	3.73	2.81	Clostridiales	-	0.002
OTU_35	11.11	118.93	45.14	10.27	Clostridiales	Ruminococcus gnavus	0.004
OTU_316	10.72	0.45	3.68	2.85	Clostridiales	Dialister sp	0.005
OTU_1053	10.57	1.76	0.59	0.96	Clostridiales	-	0.005
OTU_448	10.16	1.31	0.00	0.46	Clostridiales	-	0.006
OTU_126	10.07	5.62	2.50	22.54	Bacteroidales	Bacteroides sp	0.007
OTU_416	9.85	0.00	0.09	0.35	Clostridiales	-	0.007
OTU_472	9.69	0.24	1.59	0.00	Bacteroidales	Prevotella copri	0.008
OTU_674	9.56	0.76	0.00	0.23	Gemellales	-	0.008
OTU_491	9.30	0.24	0.41	0.00	Clostridiales	Clostridium sp	0.010
OTU_937	9.08	0.31	0.05	0.46	Bacteroidales	Butyricimonas sp	0.011
OTU_11	8.41	10.76	98.64	154.00	Clostridiales	Dialister sp	0.015
OTU_341	8.20	0.41	0.14	1.04	Clostridiales	Oscillospira sp	0.017
OTU_133	8.13	13.97	1.68	0.92	Bacteroidales	Bacteroides sp	0.017
OTU_236	7.88	0.14	2.23	2.08	Clostridiales	-	0.019
OTU_407	7.84	1.31	5.18	6.08	Clostridiales	Lachnospira sp	0.020
OTU_606	7.84	2.03	23.32	40.65	Clostridiales	Dialister sp	0.020
OTU_771	7.78	0.21	0.45	1.00	Clostridiales	Lachnospira sp	0.020
OTU_5	7.70	74.86	137.23	86.77	Clostridiales	Lachnospira sp	0.021

OTU_219	7.56	1.69	3.00	8.46	Clostridiales	-	0.023
OTU_498	7.39	1.97	1.95	1.31	Clostridiales	Lachnospira sp	0.025
OTU_383	7.27	63.90	135.41	89.54	Clostridiales	Faecalibacterium prausnitzii	0.026
OTU_1269	7.21	0.03	0.36	0.35	Clostridiales	-	0.027
OTU_83	7.19	1.17	2.82	13.73	Coriobacteriales	-	0.027
OTU_1066	7.10	0.03	0.27	0.50	Burkholderiales	Oxalobacter formigenes	0.029
OTU_549	6.92	0.14	0.05	0.54	Clostridiales	Dehalobacterium sp	0.031
OTU_100	6.80	79.24	137.14	83.00	Clostridiales	Roseburia sp	0.033
OTU_1308	6.80	8.93	16.36	7.85	Clostridiales	-	0.033
OTU_264	6.66	1.83	0.64	0.65	Clostridiales	Dorea sp	0.036
OTU_870	6.63	0.72	1.59	0.88	Clostridiales	-	0.036
OTU_633	6.60	1.86	1.82	1.23	Coriobacteriales	-	0.037
OTU_58	6.53	7.24	11.09	21.46	Bifidobacteriales	Bifidobacterium sp	0.038
OTU_6	6.46	124.72	222.41	176.08	Clostridiales	-	0.040
OTU_1259	6.45	0.48	0.95	0.54	Clostridiales	-	0.040
OTU_376	6.43	22.07	12.05	10.04	Clostridiales	Dorea sp	0.040
OTU_663	6.40	0.28	0.05	0.12	Clostridiales	Coprococcus sp	0.041
OTU_1238	6.33	5.41	3.36	1.65	Clostridiales	-	0.042
OTU_970	6.10	0.00	0.18	0.27	Clostridiales	-	0.047
OTU_43	6.10	7.90	23.86	7.35	Clostridiales	-	0.047
OTU_47	6.08	14.59	17.95	7.00	Coriobacteriales	-	0.048
OTU_143	6.03	5.38	0.41	6.58	Coriobacteriales	-	0.049

Table 7.3. Table of OTUs which were statistically significant in distribution between the subset of pre-treatment CRC samples and relatives and spouses.

OTUs with higher abundance in CRC subjects are highlighted grey. Relative abundances and p values included.

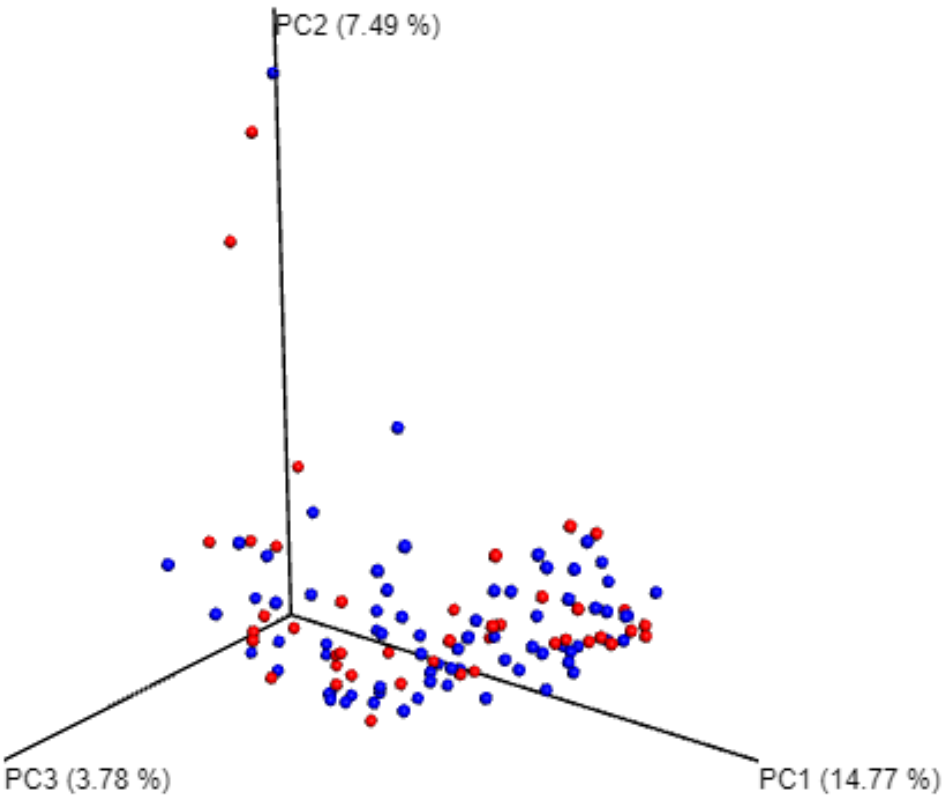


Figure 7.2. Principle component analysis of the overall diversity of pre-treatment CRC (red) and healthy controls (blue).

OTU	taxonoic order/family	Species
OTU_11	Clostridiales	Dialister sp
OTU_35	Clostridiales	Ruminococcus gnavus
OTU_83	Coriobacteriales	-
OTU_95	Clostridiales	Peptostreptococcus anaerobius
OTU_126	Bacteroidales	Bacteroides sp
OTU_219	Clostridiales	-
OTU_220	Clostridiales	Parvimonas sp
OTU_236	Clostridiales	-
OTU_264	Clostridiales	Dorea sp
OTU_213	Clostridiales	-
OTU_316	Clostridiales	Dialister sp
OTU_383	Clostridiales	Faecalibacterium prausnitzii
OTU_407	Clostridiales	Lachnospira sp
OTU_416	Clostridiales	-
OTU_448	Clostridiales	-
OTU_606	Clostridiales	Dialister sp
OTU_674	Gemellales	-
OTU_760	Fusobacteriales	Fusobacterium sp
OTU_771	Clostridiales	Lachnospira sp
OTU_970	Clostridiales	-
OTU_1053	Clostridiales	-
OTU_1269	Clostridiales	-

Table 7.4. OTUs showing statistically significant aletered abundance in both whole group and subset analysis of pre-treatment CRC subjects and controls. Those with higher abundance in CRC are highlighted in grey.

OTU	Test-Statistic	Relative mean	Spouse mean	taxonomic order/family	species	P
OTU_1224	7.66	8.67	3.82	Bacteroidales	Bacteroides sp	0.006
OTU_90	6.99	5.61	4.89	Clostridiales	-	0.008
OTU_250	6.97	1.73	0.32	Clostridiales	-	0.008
OTU_126	6.91	20.79	4.53	Bacteroidales	Bacteroides sp	0.009
OTU_133	6.83	0.76	2.61	Bacteroidales	Bacteroides sp	0.009
OTU_491	6.63	0.00	0.37	Clostridiales	Clostridium sp	0.010
OTU_75	6.59	5.39	0.68	Clostridiales	-	0.010
OTU_1086	6.25	0.03	0.34	Clostridiales	Ruminococcus sp	0.012
OTU_448	6.25	0.42	0.03	Clostridiales	-	0.012
OTU_143	6.11	5.61	0.34	Clostridiales	-	0.013
OTU_28	5.92	48.67	21.11	Clostridiales	-	0.015
OTU_911	5.85	0.33	0.71	Clostridiales	-	0.016
OTU_520	5.61	0.00	0.16	Actinomycetales	Rothia dentocariosa	0.018
OTU_472	5.31	0.03	1.00	Bacteroidales	Prevotella copri	0.021
OTU_808	5.18	0.03	0.37	Clostridiales	-	0.023
OTU_341	5.17	0.88	0.26	Clostridiales	Oscillospira sp	0.023
OTU_457	5.05	0.82	0.32	Clostridiales	-	0.025
OTU_359	4.97	0.64	0.03	Clostridiales	-	0.026
OTU_100	4.67	91.42	131.68	Clostridiales	Roseburia sp	0.031
OTU_96	4.53	4.70	0.61	Lactobacillales	Enterococcus sp	0.033
OTU_6	4.28	156.18	202.74	Clostridiales	-	0.038
OTU_318	4.23	0.39	0.29	Clostridiales	-	0.040
OTU_1060	4.17	0.55	1.50	Clostridiales	-	0.041
OTU_43	4.05	11.15	26.21	Clostridiales	-	0.044
OTU_58	3.95	17.58	12.05	Bifidobacteriales	Bifidobacterium sp	0.047

Table 7.5. Table of OTUs which were statistically significant in distribution between the relative and spouse control groups. OTUs with higher abundance in relatives are highlighted grey. Relative abundances and p values included.

7.4. Discussion

The microbial 16s profiling of pre-treatment CRC subjects, and healthy controls, comprising relatives and spouses was performed using Illumina 16s sequencing and bioinformatics analysis. This revealed that there was no significant difference for >93% of the bacterial OTUs identified between the cancer subjects and controls.

Of the identified 1346 OTUs, only 82 (6.2%) were significantly different between the CRC patients and controls, with 46 (56%) more common among the CRC subjects. Of these, 31 (67%) were identified as clostridales. There were 36 OTUs which were more common among the controls, with 33 (92%) identified as clostridales. The most predominant clostridiales identified was ruminoccus sp. with 16/82 (19.5%) OTUs characterised as such.

Other orders with higher representation among the CRC subjects include the fusobacteroides and coriobacteroides. This supports the finding of previous studies which have found an increase in the relative abundance of these species in CRC microbiomes (214, 221, 229, 245).

Subset analysis of only CRC samples which had a relative or spouse pair revealed 45 OTUs with statistically significant differences. Again, these were predominantly clostridiales. When comparing the 2 analyses there were 22 OTUs which were present in both analyses. Of these 22, 8 (36%) showed enhanced abundance among the CRC subjects, with the rest showing reduced abundance relative to the control groups. These OTUs were again identified as predominantly Clostridiales,

Coriobacteriales, Bacteroidales and Fusobacteriales, as previously reported (214, 221, 229, 245).

Interestingly a large proportion of the OTUs with an increased expression among the healthy controls were also clostridales. This suggests that any contribution of an altered microbiome towards CRC carcinogenesis or progression is more likely to be related to a "field effect" of altered metabolism and microenvironment rather than species specific changes. Of the models proposed for any potential role of the microbiome in CRC generation, these results would appear to support the "intestinal microbiota adaptions" model (238-240). This model postulates that CRC and dysbiosis may have a symbiotic relationship. The CRC environment is characterised by host-derived immune and inflammatory processes that would affect microbial regulation. The model proposes that altered immune and inflammatory processes brought about by dysplasia or mutations, could, potentially, alter the microbiome composition and favour the proliferation of pro-carcinogenic bacteria, thus amplifying the effect of dysbiosis, and further promoting CRC. It is unclear what effect diet could have on these changes, as little is known about whether the dietary factors themselves are linked to CRC development or rather the effects of the dietary factors on the microbiome.

Given that for the most part the studies to date have identified recurrent bacterial differences in CRC to order or family level, rather than specific species this would be more in keeping with a generalised alteration of microbial metabolism. These changes could be related to a more anaerobic environment, as clostridales are

obligate anaerobes. This may be less likely given the finding here that clostridiales can also be found at higher levels in healthy controls.

The relative abundance and PCA plots of the CRC samples with the healthy controls show tha the overall composition of the microbiome is very similar, indeed the ANOSIM analysis returned an R value of 0.067 (p=0.001). There are still subtle differences within the microbiome composition, but mostly not to significant levels. This could support the findings of previous studies of subtle alterations in the CRC microbiome (214, 221, 229, 245) and be in-keeping with the theory of dysbiosis in the context of CRC, or it could represent normal population variation of the microbiome with underpowering preventing any microbiome differences from being apparent. Fewer stool samples were returned by recruited subjects than urine samples, again reflecting the lower uptake of tests involving faecal matter. This led to smaller sample sizes for each of the three main groups than for the urine experiments, given the observed loss of statistical significance in Chapter 5 when the CRC urine sample cohort was reduced, this could well have resulted in underpowering.

Comparison of the two control groups, relatives and spouses, revealed that >95% of the microbiome was not statistically different. Of the identified 567 OTUs, 25 (4.4%), were shown to be statistically significantly different in relative abundance between the two control groups. There were 14 OTUs with increased abundance in relatives and 11 OTUs in spouses. Of these different OTUs, 18 (72%) were clostridiales, with 10/14 increased OTUs in the relatives and 8/11 in the spouses

identified as such. This could represent normal variation of the microbiome profile within the general population.

Further studies are required to determine the extent of the alteration of the microbiome in CRC, in particularly with regards to whether the microbiome appears to be affected by genetics or environment. Larger studies would also be needed to avoid any potential underpowering of experiments. This study does seem to confirm some of the previous work into the microbiome in CRC, in regards to altered clostridiales population and subtly different, although not to stastictically significant levels, of bacterial diversity.

CHAPTER 8

Stool 16s RNA profiling of colorectal cancer patients prior to treatment and after treatment

8.1. Introduction

As an extension of the microbiome profiling of pre-treatment CRC patients described in Chapter 7, the microbiome of the CRC patients were also studied in post-treatment samples, which were collected at 3 and 6 month intervals, after the subjects had undergone their initial treatment. This was to attempt to determine whether the microbiome alters after the primary cancer has been removed.

The aim of this study was to assess the microbiome in CRC patients and determine if successful treatment leads to any significant changes in the microbiome profile.

Further analysis was also carried out to determine whether site of CRC (right sided, left sided, or rectal) affected the microbiome composition.

8.2. Methods

CRC patients were recruited as described in section 3.2.1. Sample collection and storage was performed as described in section 3.2.4. The samples were frozen at -80°C as soon as possible after their receipt. They were transferred to the University of Warwick in dry ice and, again, stored frozen at -80°C. Samples were thawed and analysed using 16s RNA sequencing on an Illumina MiSeq platform, as described in section 3.3.3. The analysis of the .fastq sequence files from the Miseq were processed and analysed as described in chapter 3.3.3.7, using UPARSE and QIIME software and pipeline commands.

8.3. Results

As for the experiment outlined in Chapter 7, 44 CRC patients returned pre-operative samples. Of these, 15 returned samples after 3 months and 14 returned samples after 6 months.

The mean age of the pre-treatment group was 65.3 years (SD 11.2). For the 3 month post-treatment samples it was 63.2 (SD 12.1), and 66.9 (SD11.4) for the 6 month post-treatment samples. There was no statistically significant difference between the ages of the groups using ANOVA analysis, $p = 0.69$.

There were 25 males and 19 females with CRC in the pre-treatment cohort; with 8 males and 7 females in the 3 month post-treatment cohort, 7 males and 7 females in the 6 month post-treatment cohort. The male: female distribution was analysed using the Chi squared test, and found to not be significantly different; chi squared statistic 0.21, $p = 0.90$.

The average cigarette consumption per day was 0.73 (SD 2.6) for pre-treatment CRC patients, 0.67 (SD 2.5) for the 3 month post-treatment cohort and 0.7 (SD 2.6) for the 6 month post-treatment cohort. There was no statistically significant difference using ANOVA analysis, $p = 1$.

Average alcohol consumption (units per week) was 7.8 (SD 11.6) for pre-treatment CRC patients, 6.1 (SD 11.2) for the 3 month post-treatment cohort and 7.2 (SD 12.5) for the 6 month post-treatment cohort. There was no statistically significant difference using ANOVA testing, $p = 0.91$.

No CRC subject had received recent courses of antibiotics prior to providing either pre-treatment or post-treatment samples. They had not undergone recent endoscopic investigation, so had not consumed bowel preparation medications.

Mean BMI for the pre-treatment CRC cohort was 27.4 (SD 4.3), 28.5 (SD 7.7) for the 3 month post-treatment cohort and 26.8 (SD 4.6) for the 6 month post-treatment cohort. There was no statistically significant difference using ANOVA testing, $p = 0.61$.

The Duke's stages of the various groups are shown in table 8.1. There was no statistically significant difference seen between the groups using Chi squared analysis; chi squared statistic 0.67, $p = 0.99$.

The anatomical distribution of the various groups is shown in table 8.2. There was no statistically significant difference seen between the groups using Chi squared analysis; chi squared statistic 3.7, $p = 0.45$.

Duke's Stage	Pre-treatment CRC	3/12 CRC	6/12 CRC
A (%)	9 (21.4%)	3 (20.0%)	3 (21.4%)
B (%)	15 (35.7%)	5 (33.3%)	5 (35.7%)
C1 (%)	13 (30.1%)	5 (33.3%)	6 (42.9%)
C2 (%)	5 (11.9%)	2 (13.3%)	0 (0%)

Table 8.1. Duke's stages of the pre-treatment CRC group, the 3/12 and 6/12 samples of the same group.

Anatomical distribution	Pre-treatment CRC	3/12 CRC	6/12 CRC
Right sided (%)	18 (42.9%)	9 (60%)	9 (64.3%)
Left sided (%)	13 (31.0%)	4 (26.7%)	4 (28.6%)
Rectal (%)	11 (26.2%)	2 (13.3%)	1 (7.1%)

Table 8.2. Anatomical distribution of the pre-treatment CRC group, the 3/12 and 6/12 samples of the same group.

The 16s microbiome OTU data was analysed as described in section 3.3.3.7. Once the raw sequence data had been merged, quality controlled and filtered to exclude low quality reads, 10 samples had been excluded. This left remaining sample sizes of 42 for pre-treatment CRC, 15 3 month samples and 14 6 month samples.

Relative abundance plots of the pre-treatment samples compared with pooled post treatment samples can be found in Figure 8.1

The QIIME script *identify_paired_differences.py* was used to compare the pre and post operative samples. This script runs a Bonferroni corrected T-Test on each BIOM table observation to determine if the mean of each distribution of pre/post differences differs from zero.

There were 567 OTUs identified across pre-treatment and 3 and 6 month post-treatment samples.

Of these 17 (3.0%) were found to be statistically significantly different between the pre-treatment and 3 month post-treatment group. Of these 11 were more abundant in the pre-treatment samples and 6 were more abundant in the post-treatment samples. These are outlined in table 8.1. Figure 8.2 contains plots of OTUs 213 and 542, which had increased in abundance post-treatment and OTUs 44 and 627 which decreased in abundance post-treatment. Figure 8.3 shows a rarefaction curve with an average of the number of OTUs and sequences per sample. This shows an increase in the number of observed OTUs post-treatment compared to pre-treatment, but not to statistical significance.

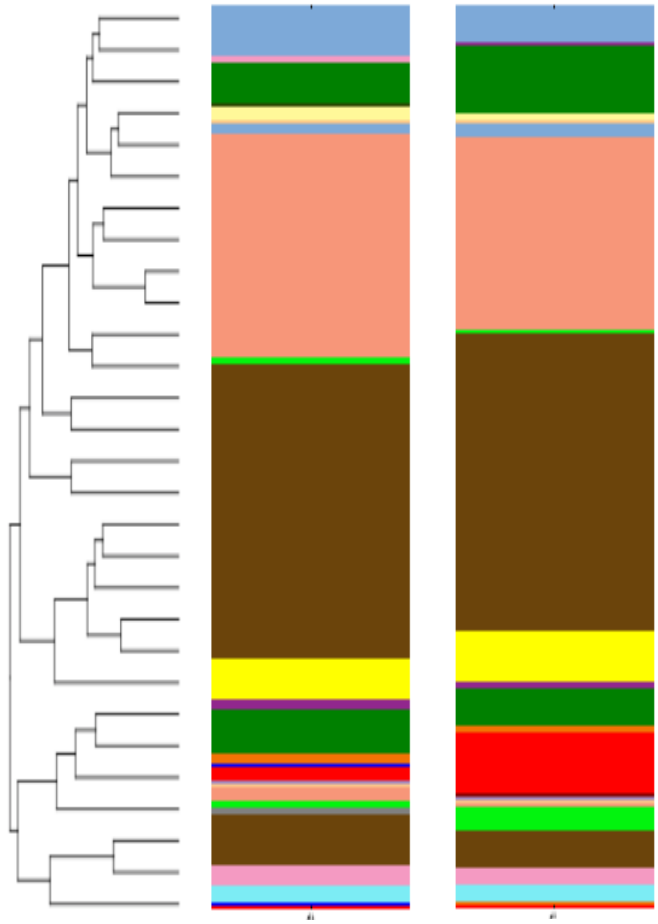


Figure 8.1. Relative abundance plots of pre-treatment CRC subjects (left) and all post op samples (right).

OTU	Test-Statistic	Pre-treatment mean	3 month post-treatment mean	Taxonomy order/family	species	P
OTU_50	2.39	15.40	2.07	Ruminococcaceae	-	0.005
OTU_87	2.73	0.93	0.00	Rikenellaceae	-	0.006
OTU_171	-2.29	0.07	2.50	Lactobacillaceae	Lactobacillus zeae	0.007
OTU_65	2.64	5.67	0.64	Ruminococcaceae	Oscillospira sp	0.012
OTU_235	-2.26	0.53	22.29	Clostridiales	-	0.017
OTU_44	2.21	23.80	5.00	Clostridiales	Roseburia sp	0.020
OTU_542	-2.55	0.00	0.50	Clostridiales	-	0.024
OTU_278	2.14	0.93	0.14	Coriobacteriaceae	-	0.025
OTU_92	1.68	4.00	0.00	Bacteroidales	Bacteroides fragilis	0.026
OTU_213	-1.85	0.47	4.50	Clostridiales	-	0.028
OTU_627	2.37	4.93	1.14	Clostridiales	-	0.030
OTU_29	-1.44	17.87	187.36	Streptococcaceae	Streptococcus sp	0.034
OTU_73	1.98	5.73	1.00	Bacteroidales	Bacteroides sp	0.036
OTU_939	2.21	1.87	0.36	Clostridiales	Ruminococcus sp	0.036
OTU_220	1.44	16.07	0.00	Clostridiales	Parvimonas sp	0.040
OTU_116	-1.62	0.13	16.57	Clostridiales	Veillonella dispar	0.043
OTU_118	1.90	4.27	1.14	Clostridiales	Ruminococcus sp	0.049

Table 8.3. Table of OTU which were statistically significant in distribution between the pre-treatment and 3 month post-treatment samples. OTUs with higher abundance in pre-treatment CRC subjects are highlighted grey. Relative abundances and p values included.

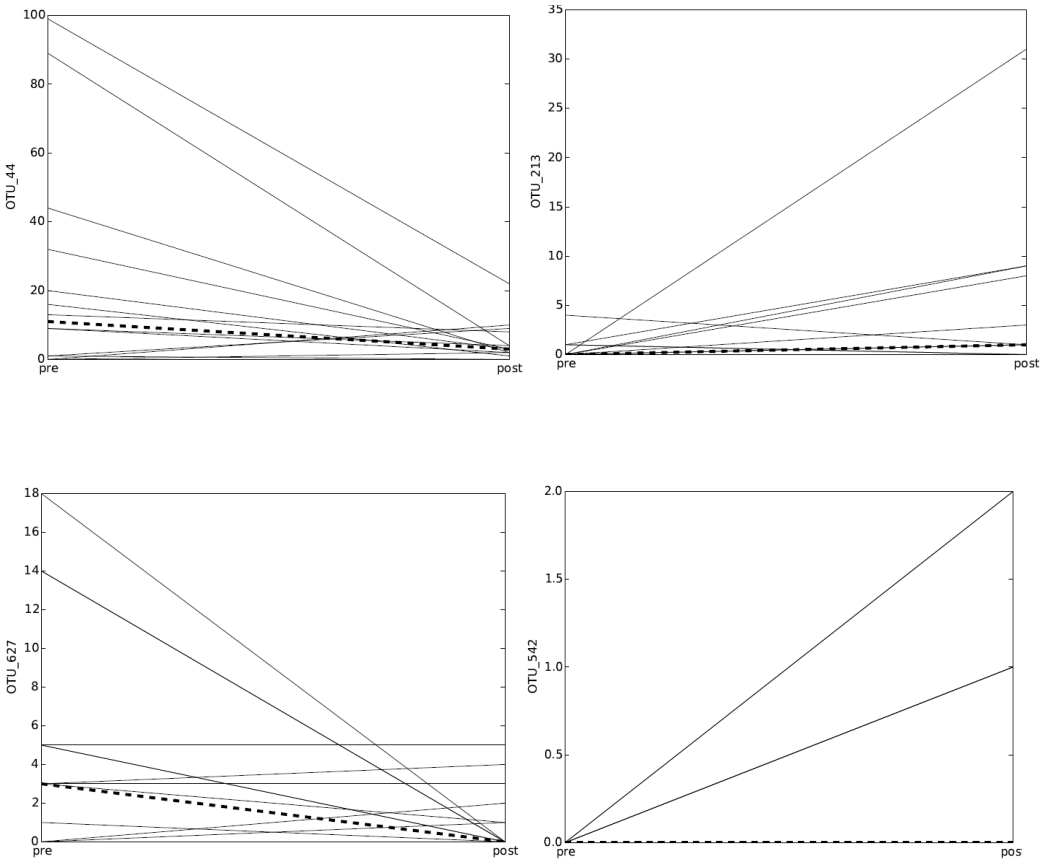


Figure 8.2. Plots of mean abundances pre and post-treatment at 3 months for OTUs 213 and 542, which had increased in abundance post-treatment and OTUs 44 and 627 which decreased in abundance post-treatment.

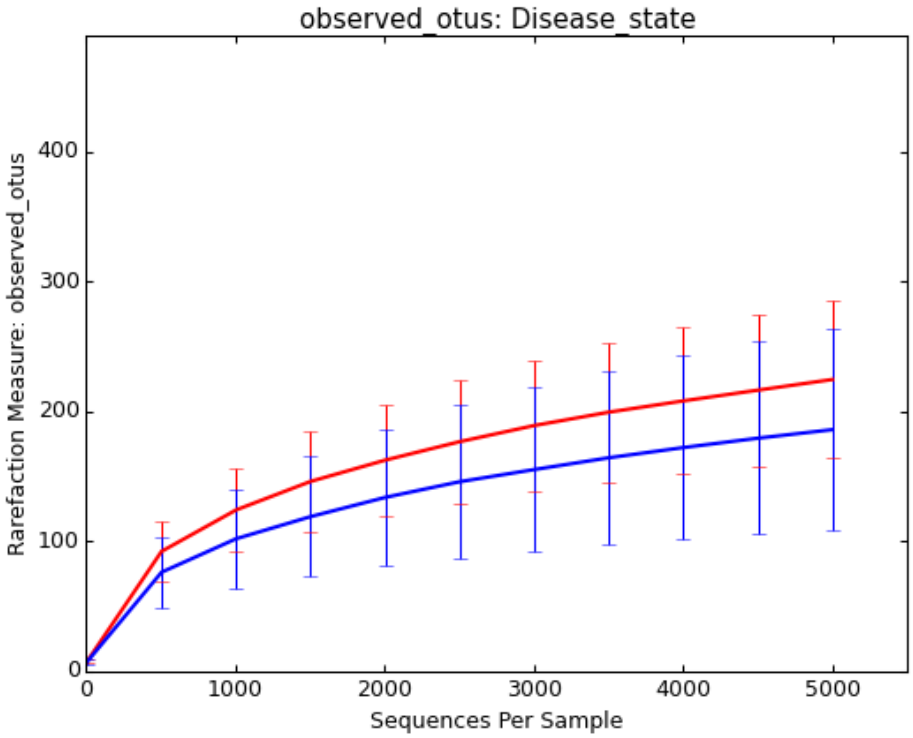


Figure 8.3. Rarefaction curve of observed number of OTUs against sequences per sample for pre-treatment samples (blue) and 3 month post-treatment samples (red).

The same analysis was then performed between the pre-treatment samples and the 6 mont post-treatment samples. Of the 567 OTUs, 22 (4.0%) were found to be statistically significantly different between the pre-treatment and 6 month post-treatment group. Of these 21 were more abundant in the pre-treatment samples and only 1 (OTU 213) was more abundant in the post-treatment samples. These are outlined in table 8.2. Figure 8.4 contains plots of OTUs 213, which had increased in abundance post-treatment and OTUs 240, 278 and 1145, which all decreased in abundance post-treatment. Figure 8.5 shows a rarefaction curve with an average of the number of OTUs and sequences per sample. This shows an increase in the number of observed OTUs post-treatment at 6 months compared to pre-treatment, but not to statistically significance.

A comparison of OTUs according to site of CRC; right, left or rectal, was performed. This showed that there were 20 OTUs (3.5%) which had statistically significant levels of abundance depending on the site of CRC. See table 8.3. Of these, 4 were predominant in right sided lesions, 5 in left sided and 8 in rectal cancers. The remaining 3 were equally abundant in 2 areas, but significantly more so than the remaining region.

OTU	Test-Statistic	Pre-treatment mean	6 month post treatment mean)	Taxonomy order/ family	Species	P
OTU_95	1.08	70.62	0.07	Clostridiales	Peptostreptococcaceae sp	0.008
OTU_511	3.37	0.77	0.07	Clostridiales	Ruminococcus sp	0.008
OTU_110	2.38	5.31	0.43	Clostridiales	-	0.010
OTU_308	1.59	3.92	0.50	Clostridiales;	Ruminococcus gnavus	0.012
OTU_37	1.93	13.62	2.93	Bacteroidales	-	0.014
OTU_455	2.85	0.38	0.00	Clostridiales		0.017
OTU_220	1.24	14.85	0.57	Clostridiales	Parvimonas sp	0.021
OTU_79	1.77	6.77	0.79	Coriobacteriales	Eggerthella lenta	0.023
OTU_33	1.95	31.92	7.57	Clostridiales	Dorea sp	0.025
OTU_278	2.36	0.69	0.07	Coriobacteriaceae	-	0.026
OTU_240	2.15	2.00	0.64	Clostridiales	Coprococcus sp	0.028
OTU_42	1.81	33.62	10.86	Clostridiales	Dorea formicigenerans	0.031
OTU_213	-1.86	0.31	5.71	Clostridiales	-	0.031
OTU_1145	1.94	1.85	0.36	Clostridiales	-	0.031
OTU_247	2.35	1.62	0.36	Clostridiales	-	0.035
OTU_1039	1.97	0.23	0.00	Clostridiales	-	0.035
OTU_412	1.45	0.85	0.00	Bacteroidales	-	0.037
OTU_488	2.30	0.54	0.00	Clostridiales	-	0.038
OTU_1237	1.90	1.46	0.14	Clostridiales	-	0.041
OTU_620	1.88	2.77	0.43	Bacteroidales	Bacteroides sp	0.043
OTU_1	1.46	238.85	25.57	Verrucomicrobiales	Akkermansia muciniphila	0.044
OTU_772	1.36	2.08	0.07	Lactobacillales	Enterococcus sp	0.048

Table 8.4. Table of OTU which were statistically significant in distribution between the pre-treatment and 6 month post-treatment samples. OTUs with higher abundance in pre-treatment CRC subjects are highlighted grey. Relative abundances and p values included.

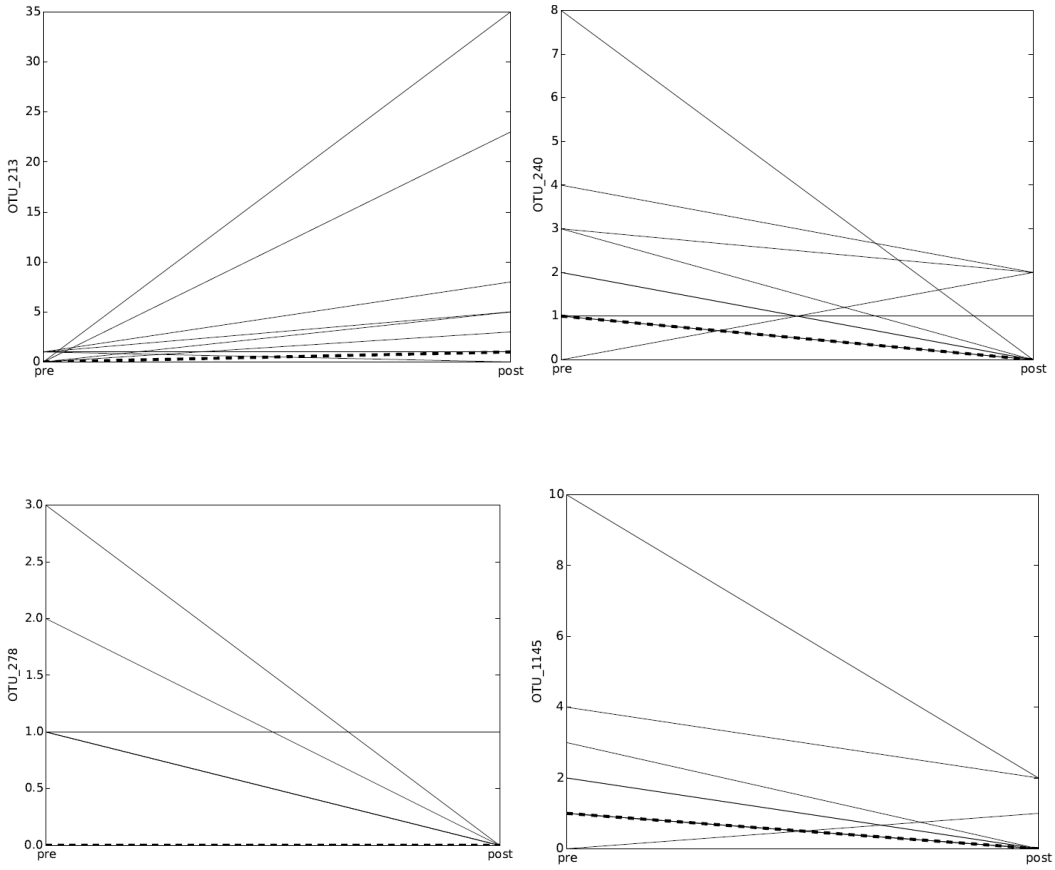


Figure 8.4. Plots of mean abundances pre and post-treatment at 6 months for OTUs 213, which had increased in abundance post-treatment, and OTUs 240, 278 and 1145, which all decreased in abundance post-treatment.

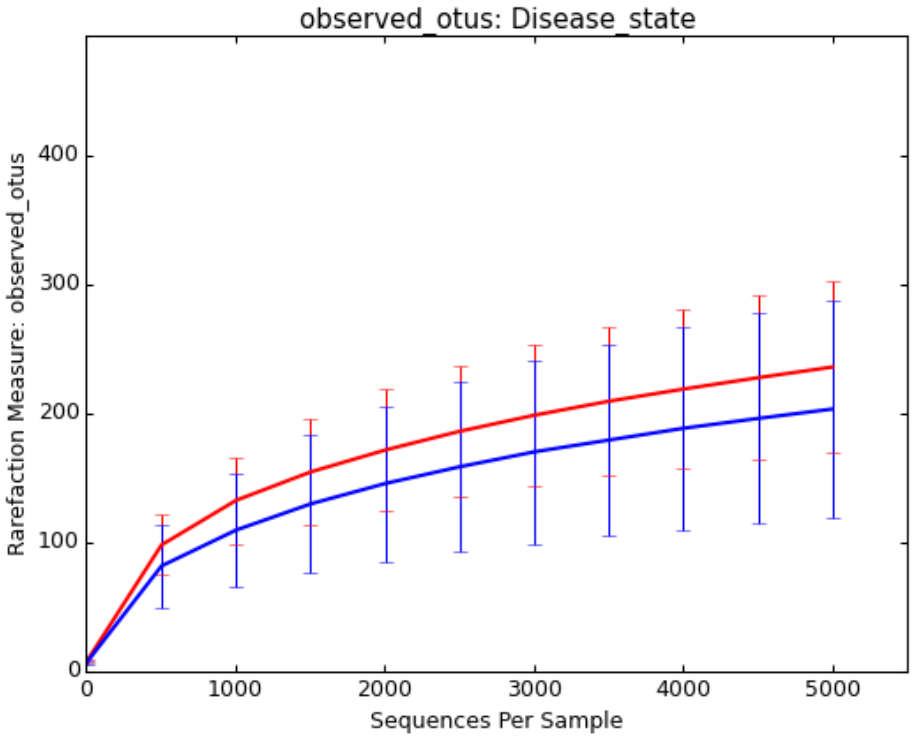


Figure 8.5. Rarefaction curve of observed number of OTUs against sequences per sample for pre-treatment samples (blue) and 6 month post-treatment samples (red).

OTU	Test-Statistic	Right_mean	Left_mean	Rectal_mean	taxonomic order/family	species	P
OTU_643	14.02	0.00	0.08	0.08	Clostridiales	-	0.003
OTU_365	14.01	0.00	0.50	0.08	Clostridiales	-	0.003
OTU_1066	11.46	0.06	0.17	0.00	Burkholderiales	Oxalobacter formigenes	0.009
OTU_160	11.20	1.12	6.33	4.67	Desulfovibrionales	Bilophila sp	0.011
OTU_921	10.60	1.06	0.00	0.75	Lactobacillales	Streptococcus sp	0.014
OTU_45	9.63	1.47	11.08	16.25	Clostridiales	-	0.022
OTU_225	9.13	0.24	0.08	0.00	Bacteroidales	Bacteroides plebeius	0.028
OTU_1	9.10	142.47	355.00	442.67	Verrucomicrobiales	Akkermansia muciniphila	0.028
OTU_530	8.85	0.12	0.00	0.83	Clostridiales	-	0.031
OTU_804	8.79	0.12	1.08	0.08	Clostridiales	Ruminococcus sp	0.032
OTU_463	8.74	0.06	0.17	0.58	Clostridiales	-	0.033
OTU_363	8.60	0.12	0.08	2.25	Clostridiales	Ruminococcus sp	0.035
OTU_36	8.39	44.24	14.92	70.75	Clostridiales	-	0.039
OTU_572	8.38	0.35	0.00	0.42	Clostridiales	-	0.039
OTU_35	8.38	75.29	174.83	16.08	Clostridiales	Ruminococcus gnavus	0.039
OTU_408	8.34	0.82	0.33	1.67	Clostridiales	-	0.040
OTU_82	8.19	6.76	4.42	15.58	Clostridiales	-	0.042
OTU_737	7.98	0.71	0.42	0.00	Clostridiales	-	0.047
OTU_22	7.86	128.29	58.58	49.08	Clostridiales	-	0.049
OTU_1122	7.85	1.59	1.58	0.08	Unassigned		0.049

Table 8.5. Table of OTUs which were statistically significant in distribution between different sites of pre-treatment CRC. OTUs with higher abundance in Right sided CRC subjects are highlighted dark grey and left sided CRC in light grey. Relative abundances and p values included.

8.4. Discussion

The microbial 16s profiling of pre and post-treatment CRC samples using Illumina 16s sequencing and bioinformatics analysis revealed that there was no significant difference for >95% of the bacterial OTUs identified.

Only 3% and 4% of the 567 identified OTUs in the 3 and 6 month post-treatment samples were significantly different from the pre-treatment samples, although there was an increase from 3 to 6 months. This supports previous work which has shown that there is a change in post-operative CRC microbiome (245, 246), although here, with the exception of 2 OTUs, there were no consistent differences with increasing time from surgery in the same patient cohort. There was a mild increase in relative abundances from 3 to 6 months post-treatment which could suggest a progressive change in the microbiome post CRC treatment, however is more likely to represent normal variation with the population. Although much longer term sample collection would be needed to confirm whether this occurs.

The rarefaction curve plots of observed OTUs, plotted gainst average number of sample reads shows an increase in the number of observed OTUs in post-treatment samples, though not to statistically significant levels. This suggests that the restriction of the microbiome, found previously by other studies (214, 218) and described in Chapter 7, could be partially reversed once the CRC has been treated, although not to significant levels.

There were 2 OTUs, 213 and 278 which were significantly different between pre-treatment samples and both 3 month and 6 month post-treatment samples. OTU

213 showed increased levels of abundance, relative to pre-treatment samples, in both 3 and 6 month post-treatment samples, whilst OTU 278 showed decreased levels of abundance in post-treatment samples compared to pre-treatment samples. OTU 213 corresponded to bacteria from the Clostridales order and OTU 278 to a bacteria from the Coriobacteriales order. Unfortunately, identification beyond the phylogenetic order was not achieved in either case. Of the 17 OTUs to show significant differences at 3 months and the 22 OTUs at 6 months, 11 and 16 respectively were Firmicutes and more specifically clostridales. Firmicutes represent approximately 10% of the colonic bacteria and are obligate anaerobes. Most of the changes observed showed a reduction in the numbers of these bacterial OTUs, although some Clostridales, such as OTU 213, did increase. This could reflect an overall less hypoxic environment within the colonic lumen in response to CRC resection. This in turn could also lead to fewer ROS and hence less potential for cellular and DNA damage as a result of anaerobic metabolism.

These findings correspond with previously reported work which has implicated a role for Clostridales species in CRC (214, 221, 245).

As discussed in Chapter 7 of the models proposed for the role of the microbiome in CRC generation, the current favoured one is the “intestinal microbiota adaptions” model (238-240). This model suggests that CRC and dysbiosis may have a symbiotic relationship. The CRC environment is characterised by host-derived immune and inflammatory processes that would affect microbial regulation. This could, potentially, alter microbiome composition and favour the proliferation of

pro-carcinogenic bacteria, thus amplifying the effect of dysbiosis, and further promoting CRC progression. This would seem more likely than the “alpha bug” and “driver/passenger” theories, as to date, there have been no bacterial species which have been consistently found to be increased in CRC. These models could still be correct, but if different bacteria were functioning as the “alpha bug” then this would suggest it is the overall effects of altered bacterial composition rather than the effects of individual bacteria.

The changes in microbiome composition, particularly with regards to those seen for OTUs 213 and 278 would need to be further investigated. It may be unlikely that these bacteria are acting as lone carcinogenic or carcinoprotective agents, but may represent more of a “field effect” found in the dysbiotic microbiome of patients with CRC. It should be noted that the sample sizes here (44, 16 and 13) were relatively small and so may be underpowered to detect any changes in the microbiome composition.

Analysis of the microbiome profiles of pre-treatment CRC by site of cancer, identified 20 OTUs, which had significantly different expression levels between the different sites. There was a roughly equal mix of those more common in right sided, left sided and rectal cancers. Again the OTUs corresponded to mainly clostridiales, although 1 could not be assigned. The sample sizes here were small, 18 right sided, 13 left and 13 rectal. This would suggest that the data is underpowered to detect any differences in microbiome profile between the different disease sites. The microbiome studied is also obtained from stool

samples obtained by defaecation, rather than from the site of the tumour. Any right sided tumour stool will have passed through the left colon and the rectum to be obtained. This means it could be contaminated by colonic micro-organisms which are not from the site of the tumour. This is an area which will require much larger sample numbers and more stringently acquired samples.

Given the relatively small sample sizes here, the lack of significant differences between the cohorts is potentially due to underpowering. Although subtle differences were observed, these were not to statistically significant levels and further larger studies would be required to determine if the microbiome does indeed alter post CRC removal. Larger samples sizes would also allow the effects of other potential confounding factors, such as chemotherapy, to be assessed. Longer follow up samples would also need to be collected to allow for better mapping of termporal changes in microbiome composition in the post operative CRC patients.

CHAPTER 9

Final Discussions

9.0 Final Discussions

The aim of this thesis was to characterise the urinary VOC profile of CRC patients and determine whether it could be distinguished from the VOC profiles of genetic and environmental controls (relatives and spouses) using an LC-FAIMS-MS apparatus, which is a variant on the FAIMS technology which has previously successfully been able to show this distinction. This was with the intention to support the growing body of evidence that urinary VOCs could distinguish CRC patients from healthy controls and potentially serve as a new, alternative, non-invasive biomarker for the disease (158-161).

The microbiome analysis via 16s RNA analysis of stool samples from the 3 groups was aimed at determining whether there was a significant difference in microbiome composition in CRC subjects compared to genetic and environmental controls. Urinary VOC and stool microbiome profiling were also performed on pre- and post-treatment CRC patient samples to determine if the urinary VOC and microbiome profiles altered in response to treatment of the CRC.

The LC-FAIMS-MS apparatus was able to show that pre-treatment CRC subjects could be distinguished from both control groups, relatives and spouses, using a 5-fold cross validation for sparse logistics regression and Random Forrest models The obtained sensitivities of 63-69%, specificities of 64-69% and AUC 0.71-0.72 are in keeping with previous work published on urinary VOC detection of CRC by a variety of different technologies (158-161). This discrimination was not found

when a smaller subset of CRC samples were compared to the control groups. This suggests that the study became underpowered by loss of these samples. The PPVs of the two models were 54 and 60%, which compare favourably to FOBT/FIT, but the NPVs of 46% and 76% compare less favourably. One of the major obstacles that would need to be overcome in the future if VOC analysis is to enter clinical practice as a screening tool for CRC would be the relatively poor performance with regards to definitively excluding disease. The relatively poor NPVs may be due to the artificial nature of the population being analysed, in that the prevalence of CRC will be far greater in a study such as this compared to a screened population. Future studies should perhaps look at recruiting far higher numbers of controls to correct for this.

As discussed in Chapter 5, the LC-FAIMS-MS technology is a variant on the FAIMS technology, which has previously been able to distinguish CRC patient urinary samples from healthy controls. The configuration used in this thesis is novel, and has not been used to investigate urinary VOCs in CRC before, although broadly based on the same technology. Not only is the technology relatively novel, but recognised statistical pathways for analysing the data do not exist. The bioinformatician who performed the analysis here reported that work on optimising the statistical method would represent a Doctor of Philosophy (PhD) thesis in its own right. Given these limitations, it is very encouraging that significant differences betwee the cohorts could be found. It should also be noted that is the first study to use healty control subjects that are connected to the CRC

subjects, by either sharing an environment, or being first degree relatives. All previous studies had used completely separate healthy controls. It is therefore also encouraging that despite the closer association of the controls used here to the cancer subjects, that they could still be distinguished with similar sensitivities, specificities and AUCs to those previously demonstrated using these techniques.

The lack of a demonstratable significant difference in VOC profile post-treatment is likely to represent underpowering of the analysis due to small sample sizes. The alternative explanations could be that the samples were collected too soon post-treatment for an effect to be observed, or that there is no change. If it is the former case, then given that the samples were collected 6 months after treatment, then this would indicate that VOC profiling would not be an ideal medium for monitoring for disease recurrence as the interval for change appears too great for accepting in clinical practice. If it is the latter then it is obviously not an appropriate method.

Profiling of the microbiome via 16s RNA analysis of stool samples from the CRC subjects and control groups identified 1346 unique OTUs across all samples. Of these >93% were similar between the CRC patients and control groups. This was backed up by an analysis of similarity result of returned an R value of 0.067 ($p=<0.001$). This indicates that whilst there is a subtle difference in the microbiome profile of patients with CRC that it is not statistically different from the variation that is seen in the general population, as the control groups had a variation of 4%. Of the identified OTUs, 82 (6.2%) were significantly different in the CRC cohort,

with 46 showing increased abundance in CRC subjects. Subset analysis of a smaller CRC patient cohort, similar to that conducted on the urinary samples, identified 45 OTUs, with 22 identified in both analyses. However, as for the urinary VOC results, given the reduction in sample size, this leads to the possibility of underpowering for the experiment and so that data must be interpreted with caution. The OTUs with significantly different abundance were identified as predominantly Clostridiales, Coriobacteriales, Bacteroidales and Fusobacteriales, as previously reported (214, 221, 229, 245).

There were differences in the microbiome of 3% and 4% respectively at 3 and 6 month post-treatment, in addition to a subtle increase, though not statistically significant, in microbial diversity. 2 specific bacterial OTUs showed significantly altered levels at both 3 and 6 months. OTU 213, a Clostridales, increased post operatively and OTU 278, a Coriobacteriales, decreased post-treatment. The alteration in these bacterial orders have been reported previously (243, 244, (247). The data on both the comparison of CRC microbiome with controls and post-treatment samples is too limited to draw conclusions about any potential microbiome role in CRC carcinogenesis, or whether the observed changes are merely an effect of CRC generation.

The original intention of this study was to recruit 200 CRC subjects, 100 relatives and 100 spouses to try and prevent underpowering of the study. This was based on previous VOC studies by our group on CRC and other diseases which had shown that sample sizes as low as 20 were able to distinguish disease groups from

controls (162, 163). Recruitment reached only approximately 50% of the intended target. This was multi-factorial, but highlights the difficulties in recruiting sizable patient numbers within the limited time window of a 2 year MD project. There was also a marked number of first degree relatives, 24, out of 61 recruited, who did not return samples. This is perhaps reflective of the fact that the study possibly, carries less importance to them, than the CRC subjects and their spouses. The final numbers for each group, 56 (CRC), 37 (relatives) and 41 (spouses) were appear to be large enough to answer the main study question with regards to urinary VOCs. However, when smaller groups were analysed, such as the CRC subset, CRC by site of tumour, or 3/6 month post-treatment samples, then the sample sizes became much smaller and the risk of underpowering increased. This can be seen in the loss of signal after a more stringently matched subset of CRC subjects was compared to relative and spouse control groups.

The data obtained does not allow us to determine whether the microbiome affects the VOC profile found in urine for either CRC subjects nor the healthy control groups. Nor does it allow us to infer whether genetic or environmental factors have a greater effect on VOC profiles, microbiome profiles or CRC generation. However, given that the healthy control groups were distinguishable from the CRC cohort using urinary VOCs, but indistinguishable from each other, it would suggest that the genetic and environmental factors although important in CRC not represented in the VOC profiles of individuals. These factors may well be beyond the scope of urinary VOCs and microbiome profiling to adequately characterise.

This thesis, as far as I am aware, provide the first work on the degradation of urinary VOCs for samples stored at room temperature prior to freezing. Previously only the effects of storage conditions on blood and faecal VOCs had been studied (241, 242). The experiments showed that urine samples show a consistent degradation pattern when stored at room temperature for increasing time intervals prior to freezing. This pattern included a plateau of degradation between 12 and 48 hours, before further degradation occurred. Interestingly it also highlighted that the samples appear to degrade within the sampling time frame of the FAIMS machine, in that the first run of a sample gave a different FAIMS matrix than the fourteenth and final run, even when stored in a chilling tray to allow automated sampling. The degradation pattern was consistent across all samples, indicating that it is a intrinsic feature of the VOCs, rather than as the result of individual sample contamination. The role of bacterial degradation is questionable here, as the degradation pattern was consistent across all subjects and samples were obtained from healthy individuals.

This work into the degradation of urinary VOCs at room temperature will hopefully add to our ever increasing understanding of VOCs and their potential role as non-invasive biomarkers of disease. It highlights how little is known about the effects of storage on VOC profile and how there is a pressing need for an agreed on standardised sample collection and storage protocols to allow comparison between different studies. This experiment has affected the ways in which further sample collection is conducted at our centre and follows up on previous work that

suggests urine samples can only be stored frozen at -80°C for up to 9 - 12 months before they degrade (248).

Potential limitations of the work presented in this thesis include, the potential for underpowering, particularly with regard to the pre and post-treatment sample analysis, as already discussed in Chapters 6 and 8. Furthermore, as with all VOC studies, the lack of an agreed upon optimum collection and storage methodology means that any results must be interpreted with caution. The degradation study conducted in Chapter 4 indicate sthat storage does affect VOC profile, and while it appears unlikely to have greatly affected the results as described here, it remains a possibility.

The results reported in this thesis will hopefully add to the growing body of work which demonstrates the detection of CRC in patients via analysis of the urinary VOC profiles. The long term goal being to find a screening test which is more acceptable to the general public than the current stool based BCSP tests of FOBT/FIT and would thusly result in a higher uptake than is currently seen. The sensitivities and specificities demonstrated are comparable to previous urinary VOC studies in CRC and to that achieved by FOBT/FIT, suggesting that if an agreed upon metholodgy could be developed for sample collection, storage and analysis that urinary VOCs could potentially be used in CRC screening programmes, although the NPV is currently too low for clinical use. Further larger studies, including larger control cohorts, are required into the use of urinary VOCs in CRC detection, particularly in direct comparison to the other screening tools used such

as FOBT/FIT to determine whether indeed urinary VOC analysis is superior in terms of accuracy and uptake. These studies would also need to have a more stringent sample collection protocol, either fresh samples, or samples within a 12-48 hour period post voiding. Ideally these would be fresh samples, given that the long term goal would be to have urinary VOC screening for CRC available as a point of care test in clinically settings.

This is the first study to utilise this precise FAIMS variant, the LC-FAIM-MS, to detect urinary VOCs in CRC patients and therefore as further work is conducted in this area then more refining of the both the LC-FAIMS-MS apparatus and statistical pathways used may yield further progress in the quest for a reliable, non-invasive, urinary biomarker for colorectal cancer.

Bibliography

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-917.

2. J F, I S, M E, R D, S E, C M, et al. Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. GLOBOCAN 2012 v10 [Internet]. 2013.

3. UK CRC. 2013 diagnoses [Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer#heading-Zero.

4. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends-An Update. Cancer Epidemiol Biomarkers Prev. 2016;25(1):16-27.

5. Jones AM, Morris E, Thomas J, Forman D, Melia J, Moss SM. Evaluation of bowel cancer registration data in England, 1996-2004. Br J Cancer. 2009;101(8):1269-73.

6. UK CR. [Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence#ref-0.

7. Scholefield JH, Eng C. Colorectal Cancer: Diagnosis and Clinical Management. 1st ed: John Wiley and Sons Ltd; 2014.

8. UK CR. 5 year survival statistics [Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/survival#heading-Three.

9. Murphy G, Devesa SS, Cross AJ, Inskip PD, McGlynn KA, Cook MB. Sex disparities in colorectal cancer incidence by anatomic subsite, race and age. Int J Cancer. 2011;128(7):1668-75.

10. Galiatsatos P, Foulkes WD. Familial adenomatous polyposis. Am J Gastroenterol. 2006;101(2):385-98.

11. Gala M, Chung DC. Hereditary colon cancer syndromes. Semin Oncol. 2011;38(4):490-9.

12. Butterworth AS, Higgins JP, Pharoah P. Relative and absolute risk of colorectal cancer for individuals with a family history: a meta-analysis. Eur J Cancer. 2006;42(2):216-27.

13. Zöller B, Li X, Sundquist J, Sundquist K. Familial transmission of prostate, breast and colorectal cancer in adoptees is related to cancer in biological but not in adoptive parents: a nationwide family study. Eur J Cancer. 2014;50(13):2319-27.

14. von Holst S, Picelli S, Edler D, Lenander C, Dalén J, Hjern F, et al. Association studies on 11 published colorectal cancer risk loci. Br J Cancer. 2010;103(4):575-80.

15. Niittymäki I, Kaasinen E, Tuupanen S, Karhu A, Järvinen H, Mecklin JP, et al. Low-penetrance susceptibility variants in familial colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2010;19(6):1478-83.

16. Dunlop MG, Dobbins SE, Farrington SM, Jones AM, Palles C, Whiffin N, et al. Common variation near CDKN1A, POLD3 and SHROOM2 influences colorectal cancer risk. Nat Genet. 2012;44(7):770-6.

17. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterology. 2010;138(6):2044-58.

18. Martínez ME, Baron JA, Lieberman DA, Schatzkin A, Lanza E, Winawer SJ, et al. A pooled analysis of advanced colorectal neoplasia diagnoses after colonoscopic polypectomy. Gastroenterology. 2009;136(3):832-41.

19. Hassan C, Gimeno-García A, Kalager M, Spada C, Zullo A, Costamagna G, et al. Systematic review with meta-analysis: the incidence of advanced neoplasia after polypectomy in patients with and without low-risk adenomas. Aliment Pharmacol Ther. 2014;39(9):905-12.

20. Lutgens MW, van Oijen MG, van der Heijden GJ, Vleggaar FP, Siersema PD, Oldenburg B. Declining risk of colorectal cancer in inflammatory bowel disease: an

updated meta-analysis of population-based cohort studies. Inflamm Bowel Dis. 2013;19(4):789-99.

21. Chiong C, Cox MR, Eslick GD. Gallstone disease is associated with rectal cancer: a meta-analysis. Scand J Gastroenterol. 2012;47(5):553-64.

22. Chiong C, Cox MR, Eslick GD. Gallstones are associated with colonic adenoma: a meta-analysis. World J Surg. 2012;36(9):2202-9.

23. Jiang Y, Ben Q, Shen H, Lu W, Zhang Y, Zhu J. Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies. Eur J Epidemiol. 2011;26(11):863-76.

24. Krämer HU, Schöttker B, Raum E, Brenner H. Type 2 diabetes mellitus and colorectal cancer: meta-analysis on sex-specific differences. Eur J Cancer. 2012;48(9):1269-82.

25. Larsson SC, Orsini N, Wolk A. Diabetes mellitus and risk of colorectal cancer: a meta-analysis. J Natl Cancer Inst. 2005;97(22):1679-87.

26. Luo W, Cao Y, Liao C, Gao F. Diabetes mellitus and the incidence and mortality of colorectal cancer: A meta-analysis of twenty four cohort studies. Colorectal Dis. 2011.

27. Wu L, Yu C, Jiang H, Tang J, Huang HL, Gao J, et al. Diabetes mellitus and the occurrence of colorectal cancer: an updated meta-analysis of cohort studies. Diabetes Technol Ther. 2013;15(5):419-27.

28. Jinjuvadia R, Lohia P, Jinjuvadia C, Montoya S, Liangpunsakul S. The association between metabolic syndrome and colorectal neoplasm: systemic review and meta-analysis. J Clin Gastroenterol. 2013;47(1):33-44.

29. Ma Y, Yang Y, Wang F, Zhang P, Shi C, Zou Y, et al. Obesity and risk of colorectal cancer: a systematic review of prospective studies. PLoS One. 2013;8(1):e53916.

30. Cong YJ, Gan Y, Sun HL, Deng J, Cao SY, Xu X, et al. Association of sedentary behaviour with colon and rectal cancer: a meta-analysis of observational studies. Br J Cancer. 2014;110(3):817-26.

31. Boyle T, Keegel T, Bull F, Heyworth J, Fritschi L. Physical activity and risks of proximal and distal colon cancers: a systematic review and meta-analysis. J Natl Cancer Inst. 2012;104(20):1548-61.

32. Aune D, Lau R, Chan DS, Vieira R, Greenwood DC, Kampman E, et al. Nonlinear reduction in risk for colorectal cancer by fruit and vegetable intake based on meta-analysis of prospective studies. Gastroenterology. 2011;141(1):106-18.

33. Tse G, Eslick GD. Cruciferous vegetables and risk of colorectal neoplasms: a systematic review and meta-analysis. Nutr Cancer. 2014;66(1):128-39.

34. Wu QJ, Yang Y, Vogtmann E, Wang J, Han LH, Li HL, et al. Cruciferous vegetables intake and the risk of colorectal cancer: a meta-analysis of observational studies. Ann Oncol. 2013;24(4):1079-87.

35. Koushik A, Hunter DJ, Spiegelman D, Beeson WL, van den Brandt PA, Buring JE, et al. Fruits, vegetables, and colon cancer risk in a pooled analysis of 14 cohort studies. J Natl Cancer Inst. 2007;99(19):1471-83.

36. Lee JE, Chan AT. Fruit, vegetables, and folate: cultivating the evidence for cancer prevention. Gastroenterology. 2011;141(1):16-20.

37. Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, et al. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. PLoS One. 2011;6(6):e20456.

38. Sandhu MS, White IR, McPherson K. Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. Cancer Epidemiol Biomarkers Prev. 2001;10(5):439-46.

39. Norat T, Lukanova A, Ferrari P, Riboli E. Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies. Int J Cancer. 2002;98(2):241-56.

40. Larsson SC, Wolk A. Meat consumption and risk of colorectal cancer: a meta-analysis of prospective studies. Int J Cancer. 2006;119(11):2657-64.

41. Fonseca-Nunes A, Jakszyn P, Agudo A. Iron and cancer risk--a systematic review and meta-analysis of the epidemiological evidence. Cancer Epidemiol Biomarkers Prev. 2014;23(1):12-31.

42. Key TJ, Appleby PN, Masset G, Brunner EJ, Cade JE, Greenwood DC, et al. Vitamins, minerals, essential fatty acids and colorectal cancer risk in the United Kingdom Dietary Cohort Consortium. Int J Cancer. 2012;131(3):E320-5.

43. Parkin DM, Boyd L. 6. Cancers attributable to dietary factors in the UK in 2010. III. Low consumption of fibre. Br J Cancer. 2011;105 Suppl 2:S27-30.

44. Aune D, Chan DS, Lau R, Vieira R, Greenwood DC, Kampman E, et al. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. BMJ. 2011;343:d6617.

45. Aune D, Lau R, Chan DS, Vieira R, Greenwood DC, Kampman E, et al. Dairy products and colorectal cancer risk: a systematic review and meta-analysis of cohort studies. Ann Oncol. 2012;23(1):37-45.

46. Keum N, Aune D, Greenwood DC, Ju W, Giovannucci EL. Calcium intake and colorectal cancer risk: dose-response meta-analysis of prospective observational studies. Int J Cancer. 2014;135(8):1940-8.

47. Carroll C, Cooper K, Papaioannou D, Hind D, Pilgrim H, Tappenden P. Supplemental calcium in the chemoprevention of colorectal cancer: a systematic review and meta-analysis. Clin Ther. 2010;32(5):789-803.

48. Weingarten MA, Zalmanovici A, Yaphe J. Dietary calcium supplementation for preventing colorectal cancer and adenomatous polyps. Cochrane Database Syst Rev. 2008(1):CD003548.

49. Ma Y, Zhang P, Wang F, Yang J, Liu Z, Qin H. Association between vitamin D and risk of colorectal cancer: a systematic review of prospective studies. J Clin Oncol. 2011;29(28):3775-82.

50. Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. Lancet Diabetes Endocrinol. 2014;2(1):76-89.

51. Yin L, Grandi N, Raum E, Haug U, Arndt V, Brenner H. Meta-analysis: Serum vitamin D and colorectal adenoma risk. Prev Med. 2011;53(1-2):10-6.

52. Gandini S, Boniol M, Haukka J, Byrnes G, Cox B, Sneyd MJ, et al. Meta-analysis of observational studies of serum 25-hydroxyvitamin D levels and colorectal, breast and prostate cancer and colorectal adenoma. Int J Cancer. 2011;128(6):1414-24.

53. Leenders M, Leufkens AM, Siersema PD, van Duijnhoven FJ, Vrieling A, Hulshof PJ, et al. Plasma and dietary carotenoids and vitamins A, C and E and risk of colon and rectal cancer in the European Prospective Investigation into Cancer and Nutrition. Int J Cancer. 2014;135(12):2930-9.

54. Li P, Xu J, Shi Y, Ye Y, Chen K, Yang J, et al. Association between zinc intake and risk of digestive tract cancers: a systematic review and meta-analysis. Clin Nutr. 2014;33(3):415-20.

55. Huxley RR, Ansary-Moghaddam A, Clifton P, Czernichow S, Parr CL, Woodward M. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. Int J Cancer. 2009;125(1):171-80.

56. Liang PS, Chen TY, Giovannucci E. Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. Int J Cancer. 2009;124(10):2406-15.

57. Fedirko V, Tramacere I, Bagnardi V, Rota M, Scotti L, Islami F, et al. Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. Ann Oncol. 2011;22(9):1958-72.

58. Baron JA, Sandler RS, Bresalier RS, Quan H, Riddell R, Lanas A, et al. A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. Gastroenterology. 2006;131(6):1674-82.

59. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. Lancet Oncol. 2012;13(5):518-27.

60. Cole BF, Logan RF, Halabi S, Benamouzig R, Sandler RS, Grainge MJ, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. J Natl Cancer Inst. 2009;101(4):256-66.

61. Chan AT, Giovannucci EL. Primary prevention of colorectal cancer. Gastroenterology. 2010;138(6):2029-43.e10.

62. Grodstein F, Newcomb PA, Stampfer MJ. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. Am J Med. 1999;106(5):574-82.

63. Hébert-Croteau N. A meta-analysis of hormone replacement therapy and colon cancer in women. Cancer Epidemiol Biomarkers Prev. 1998;7(8):653-9.

64. Nanda K, Bastian LA, Hasselblad V, Simel DL. Hormone replacement therapy and the risk of colorectal cancer: a meta-analysis. Obstet Gynecol. 1999;93(5 Pt 2):880-8.

65. Green J, Czanner G, Reeves G, Watson J, Wise L, Roddam A, et al. Menopausal hormone therapy and risk of gastrointestinal cancer: nested case-control study within a prospective cohort, and meta-analysis. Int J Cancer. 2012;130(10):2387-96.

66. Bosetti C, Bravi F, Negri E, La Vecchia C. Oral contraceptives and colorectal cancer risk: a systematic review and meta-analysis. Hum Reprod Update. 2009;15(5):489-98.

67. Lee JE, Willett WC, Fuchs CS, Smith-Warner SA, Wu K, Ma J, et al. Folate intake and risk of colorectal cancer and adenoma: modification by time. Am J Clin Nutr. 2011;93(4):817-25.

68. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. JAMA. 2007;297(21):2351-9.

69. Logan RF, Grainge MJ, Shepherd VC, Armitage NC, Muir KR, Group uT. Aspirin and folic acid for the prevention of recurrent colorectal adenomas. Gastroenterology. 2008;134(1):29-38.

70. Stevens A, Lowe J. Pathology. Mosby; 2000. p. 259-64.

71. UK CR. Anatomical site [Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence#heading-Five.

72. Colledge N, Walker B, Ralston S. Davidson's Principles and Practice of Medicine. 21st edition ed: Chruchill Livingstone Elsevier; 2010.

73. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. J Gastrointest Oncol. 2012;3(3):153-73.

74. DENOIX P. [Not Available]. Bull Inst Natl Hyg. 1946;1:12-7.

75. Dukes C. The Classification of Cancer of the Rectum. J Path Bad; 1932. p. 325-32.

76. Rao AR, Kagan AR, Chan PM, Gilbert HA, Nussbaum H, Hintz BL. Patterns of recurrence following curative resection alone for adenocarcinoma of the rectum and sigmoid colon. Cancer. 1981;48(6):1492-5.

77. Böhm B, Schwenk W, Hucke HP, Stock W. Does methodic long-term follow-up affect survival after curative resection of colorectal carcinoma? Dis Colon Rectum. 1993;36(3):280-6.

78. Sargent DJ, Wieand HS, Haller DG, Gray R, Benedetti JK, Buyse M, et al. Disease-free survival versus overall survival as a primary end point for adjuvant colon cancer studies: individual patient data from 20,898 patients on 18 randomized trials. J Clin Oncol. 2005;23(34):8664-70.

79. Manfredi S, Bouvier AM, Lepage C, Hatem C, Dancourt V, Faivre J. Incidence and patterns of recurrence after resection for cure of colonic cancer in a well defined population. Br J Surg. 2006;93(9):1115-22.

80. Cairns SR, Scholefield JH, Steele RJ, Dunlop MG, Thomas HJ, Evans GD, et al. Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). Gut. 2010;59(5):666-89.

81. Engstrom PF, Arnoletti JP, Benson AB, Chen YJ, Choti MA, Cooper HS, et al. NCCN Clinical Practice Guidelines in Oncology: colon cancer. J Natl Compr Canc Netw. 2009;7(8):778-831.

82. Plumb AA, Halligan S. Colorectal cancer screening. Semin Roentgenol. 2015;50(2):101-10.

83. Logan RF, Patnick J, Nickerson C, Coleman L, Rutter MD, von Wagner C, et al. Outcomes of the Bowel Cancer Screening Programme (BCSP) in England after the first 1 million tests. Gut. 2012;61(10):1439-46.

84. Bevan R, Rubin G, Sofianopoulou E, Patnick J, Rees CJ. Implementing a national flexible sigmoidoscopy screening program: results of the English early pilot. Endoscopy. 2015;47(3):225-31.

85. Rabeneck L, Rumble RB, Thompson F, Mills M, Oleschuk C, Whibley A, et al. Fecal immunochemical tests compared with guaiac fecal occult blood tests for population-based colorectal cancer screening. Can J Gastroenterol. 2012;26(3):131-47.

86. Steele RJ, McDonald PJ, Digby J, Brownlee L, Strachan JA, Libby G, et al. Clinical outcomes using a faecal immunochemical test for haemoglobin as a first-line test in a national programme constrained by colonoscopy capacity. United European Gastroenterol J. 2013;1(3):198-205.

87. van Rossum LG, van Rijn AF, Laheij RJ, van Oijen MG, Fockens P, van Krieken HH, et al. Random comparison of guaiac and immunochemical fecal occult blood tests for colorectal cancer in a screening population. Gastroenterology. 2008;135(1):82-90.

88. Huddy JR, Ni MZ, Markar SR, Hanna GB. Point-of-care testing in the diagnosis of gastrointestinal cancers: current technology and future directions. World J Gastroenterol. 2015;21(14):4111-20.

89. Fraser CG, Digby J, McDonald PJ, Strachan JA, Carey FA, Steele RJ. Experience with a two-tier reflex gFOBT/FIT strategy in a national bowel screening programme. J Med Screen. 2012;19(1):8-13.

90. Kuipers EJ, Rösch T, Bretthauer M. Colorectal cancer screening--optimizing current strategies and new directions. Nat Rev Clin Oncol. 2013;10(3):130-42.

91. Cole SR, Young GP, Esterman A, Cadd B, Morcom J. A randomised trial of the impact of new faecal haemoglobin test technologies on population participation in screening for colorectal cancer. J Med Screen. 2003;10(3):117-22.

92. Imperiale TF, Ransohoff DF, Itzkowitz SH, Levin TR, Lavin P, Lidgard GP, et al. Multitarget stool DNA testing for colorectal-cancer screening. N Engl J Med. 2014;370(14):1287-97.

93. Ahlquist DA, Shuber AP. Stool screening for colorectal cancer: evolution from occult blood to molecular markers. Clin Chim Acta. 2002;315(1-2):157-68.

94. Tonus C, Sellinger M, Koss K, Neupert G. Faecal pyruvate kinase isoenzyme type M2 for colorectal cancer screening: a meta-analysis. World J Gastroenterol. 2012;18(30):4004-11.

95. Schmidt K, Podmore I. Current Challenges in Volatile Organic Compounds Analysis as Potential Biomarkers of Cancer. J Biomark. 2015;2015:981458.

96. Arasaradnam RP, Covington JA, Harmston C, Nwokolo CU. Review article: next generation diagnostic modalities in gastroenterology--gas phase volatile compound biomarker detection. Aliment Pharmacol Ther. 2014;39(8):780-9.

97. Williams H, Pembroke A. Sniffer dogs in the melanoma clinic? Lancet. 1989;1(8640):734.

98. Pickel D, Manucy G, Walker D, Hall S, Walker J. Evidence for canine olfactory detection of melanoma. Applied Animal Behaviour Science. 2004;89:107-16.

99. Willis CM, Church SM, Guest CM, Cook WA, McCarthy N, Bransbury AJ, et al. Olfactory detection of human bladder cancer by dogs: proof of principle study. BMJ. 2004;329(7468):712.

100. McCulloch M, Jezierski T, Broffman M, Hubbard A, Turner K, Janecki T. Diagnostic accuracy of canine scent detection in early- and late-stage lung and breast cancers. Integr Cancer Ther. 2006;5(1):30-9.

101. Gordon RT, Schatz CB, Myers LJ, Kosty M, Gonczy C, Kroener J, et al. The use of canines in the detection of human cancers. J Altern Complement Med. 2008;14(1):61-7.

102. Horvath G, Järverud GA, Järverud S, Horváth I. Human ovarian carcinomas detected by specific odor. Integr Cancer Ther. 2008;7(2):76-80.

103. Moser E, McCulloch M. Canine scent detection of human cancers: A review of methods and accuracy. Journal of Veterinary Behaviour. 2010;5:145/52.

104. Sonoda H, Kohnoe S, Yamazato T, Satoh Y, Morizono G, Shikata K, et al. Colorectal cancer screening with odour material by canine scent detection. Gut. 2011;60(6):814-9.

105. Willis CM, Britton LE, Harris R, Wallace J, Guest CM. Volatile organic compounds as biomarkers of bladder cancer: Sensitivity and specificity using trained sniffer dogs. Cancer Biomark. 2010;8(3):145-53.

106. Cornu JN, Cancel-Tassin G, Ondet V, Girardet C, Cussenot O. Olfactory detection of prostate cancer by dogs sniffing urine: a step forward in early diagnosis. Eur Urol. 2011;59(2):197-201.

107. Taverna G, Tidu L, Grizzi F, Torri V, Mandressi A, Sardella P, et al. Olfactory system of highly trained dogs detects prostate cancer in urine samples. J Urol. 2015;193(4):1382-7.

108. Ehmann R, Boedeker E, Friedrich U, Sagert J, Dippon J, Friedel G, et al. Canine scent detection in the diagnosis of lung cancer: revisiting a puzzling phenomenon. Eur Respir J. 2012;39(3):669-76.

109. Buszewski B, Ligor T, Jezierski T, Wenda-Piesik A, Walczak M, Rudnicka J. Identification of volatile lung cancer markers by gas chromatography-mass spectrometry: comparison with discrimination by canines. Anal Bioanal Chem. 2012;404(1):141-6.

110. Amundsen T, Sundstrøm S, Buvik T, Gederaas OA, Haaverstad R. Can dogs smell lung cancer? First study using exhaled breath and urine screening in unselected patients with suspected lung cancer. Acta Oncol. 2014;53(3):307-15.

111. Arasaradnam RP, Nwokolo CU, Bardhan KD, Covington JA. Electronic nose versus canine nose: clash of the titans. Gut. 2011;60(12):1768.

112. Bjartell AS. Dogs sniffing urine: a future diagnostic tool or a way to identify new prostate cancer markers? Eur Urol. 2011;59(2):202-3.

113. Boedeker E, Friedel G, Walles T. Sniffer dogs as part of a bimodal bionic research approach to develop a lung cancer screening. Interact Cardiovasc Thorac Surg. 2012;14(5):511-5.

114. Jezierski T, Walczak M, Ligor T, Rudnicka J, Buszewski B. Study of the art: canine olfaction used for cancer detection on the basis of breath odour. Perspectives and limitations. J Breath Res. 2015;9(2):027001.

115. Silva CL, Passos M, Câmara JS. Investigation of urinary volatile organic metabolites as potential cancer biomarkers by solid-phase microextraction in combination with gas chromatography-mass spectrometry. Br J Cancer. 2011;105(12):1894-904.

116. Horvath I. Smells like cancer. Lung Cancer. 2010;68(2):127-8.

117. Smith D, Spanel P. Pitfalls in the analysis of volatile breath biomarkers: suggested solutions and SIFT-MS quantification of single metabolites. J Breath Res. 2015;9(2):022001.

118. Gordon SM, Szidon JP, Krotoszynski BK, Gibbons RD, O'Neill HJ. Volatile organic compounds in exhaled air from patients with lung cancer. Clin Chem. 1985;31(8):1278-82.

119. Preti G, Labows JN, Kostelc JG, Aldinger S, Daniele R. Analysis of lung air from patients with bronchogenic carcinoma and controls using gas chromatography-mass spectrometry. J Chromatogr. 1988;432:1-11.

120. O'Neill H, Gordon S, O'Neill M, Gibbons R, Szidon J. A computerized classification technique for screening for the presence of breath biomarkers in lung cancer. Clinical Chemistry. 1988;34(8):1613-8.

121. Phillips M, Gleeson K, Hughes JM, Greenberg J, Cataneo RN, Baker L, et al. Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study. Lancet. 1999;353(9168):1930-3.

122. Krilaviciute A, Heiss JA, Leja M, Kupcinskas J, Haick H, Brenner H. Detection of cancer through exhaled breath: a systematic review. Oncotarget. 2015;6(36):38643-57.

123. Phillips M, Cataneo RN, Cummin AR, Gagliardi AJ, Gleeson K, Greenberg J, et al. Detection of lung cancer with volatile markers in the breath. Chest. 2003;123(6):2115-23.

124. Phillips M, Altorki N, Austin JH, Cameron RB, Cataneo RN, Greenberg J, et al. Prediction of lung cancer using volatile biomarkers in breath. Cancer Biomark. 2007;3(2):95-109.

125. Phillips M, Altorki N, Austin JH, Cameron RB, Cataneo RN, Kloss R, et al. Detection of lung cancer using weighted digital analysis of breath biomarkers. Clin Chim Acta. 2008;393(2):76-84.

126. Fu XA, Li M, Knipp RJ, Nantz MH, Bousamra M. Noninvasive detection of lung cancer using exhaled breath. Cancer Med. 2014;3(1):174-81.

127. Li M, Yang D, Brock G, Knipp RJ, Bousamra M, Nantz MH, et al. Breath carbonyl compounds as biomarkers of lung cancer. Lung Cancer. 2015;90(1):92-7.

128. Sponring A, Filipiak W, Ager C, Schubert J, Miekisch W, Amann A, et al. Analysis of volatile organic compounds (VOCs) in the headspace of NCI-H1666 lung cancer cells. Cancer Biomark. 2010;7(3):153-61.

129. Hu YJ, Qiu YH, Chen EG, Ying KJ, Yu J, Wang P. [Determination of volatile organic compounds in lung cancer cell lines and lung cancer tissue]. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2010;39(3):278-84.

130. Filipiak W, Sponring A, Filipiak A, Ager C, Schubert J, Miekisch W, et al. TD-GC-MS analysis of volatile metabolites of human lung cancer and normal cells in vitro. Cancer Epidemiol Biomarkers Prev. 2010;19(1):182-95.

131. Buszewski B, Ulanowska A, Kowalkowski T, Cieśliński K. Investigation of lung cancer biomarkers by hyphenated separation techniques and chemometrics. Clin Chem Lab Med. 2012;50(3):573-81.

132. Filipiak W, Filipiak A, Sponring A, Schmid T, Zelger B, Ager C, et al. Comparative analyses of volatile organic compounds (VOCs) from patients, tumors and transformed cell lines for the validation of lung cancer-derived breath markers. J Breath Res. 2014;8(2):027111.

133. Taivans I, Bukovskis M, Strazda G, Jurka N. Breath testing as a method for detecting lung cancer. Expert Rev Anticancer Ther. 2013.

134. Kalluri U, Naiker M, Myers MA. Cell culture metabolomics in the diagnosis of lung cancer-the influence of cell culture conditions. J Breath Res. 2014;8(2):027109.

135. Machado RF, Laskowski D, Deffenderfer O, Burch T, Zheng S, Mazzone PJ, et al. Detection of lung cancer by sensor array analyses of exhaled breath. Am J Respir Crit Care Med. 2005;171(11):1286-91.

136. Dragonieri S, Annema JT, Schot R, van der Schee MP, Spanevello A, Carratú P, et al. An electronic nose in the discrimination of patients with non-small cell lung cancer and COPD. Lung Cancer. 2009;64(2):166-70.

137. McWilliams A, Beigi P, Srinidhi A, Lam S, MacAulay CE. Sex and Smoking Status Effects on the Early Detection of Early Lung Cancer in High-Risk Smokers Using an Electronic Nose. IEEE Trans Biomed Eng. 2015;62(8):2044-54.

138. Mazzone PJ, Hammel J, Dweik R, Na J, Czich C, Laskowski D, et al. Diagnosis of lung cancer by the analysis of exhaled breath with a colorimetric sensor array. Thorax. 2007;62(7):565-8.

139. Mazzone PJ, Wang XF, Xu Y, Mekhail T, Beukemann MC, Na J, et al. Exhaled breath analysis with a colorimetric sensor array for the identification and characterization of lung cancer. J Thorac Oncol. 2012;7(1):137-42.

140. Mazzone PJ, Wang XF, Lim S, Choi H, Jett J, Vachani A, et al. Accuracy of volatile urine biomarkers for the detection and characterization of lung cancer. BMC Cancer. 2015;15:1001.

141. Peng G, Tisch U, Adams O, Hakim M, Shehada N, Broza YY, et al. Diagnosing lung cancer in exhaled breath using gold nanoparticles. Nat Nanotechnol. 2009;4(10):669-73.

142. Barash O, Peled N, Hirsch FR, Haick H. Sniffing the unique "odor print" of non-small-cell lung cancer with gold nanoparticles. Small. 2009;5(22):2618-24.

143. Di Natale C, Macagnano A, Martinelli E, Paolesse R, D'Arcangelo G, Roscioni C, et al. Lung cancer identification by the analysis of breath by means of an array of non-selective gas sensors. Biosens Bioelectron. 2003;18(10):1209-18.

144. D'Amico A, Pennazza G, Santonico M, Martinelli E, Roscioni C, Galluccio G, et al. An investigation on electronic nose diagnosis of lung cancer. Lung Cancer. 2010;68(2):170-6.

145. Santonico M, Lucantoni G, Pennazza G, Capuano R, Galluccio G, Roscioni C, et al. In situ detection of lung cancer volatile fingerprints using bronchoscopic air-sampling. Lung Cancer. 2012;77(1):46-50.

146. Gasparri R, Santonico M, Valentini C, Sedda G, Borri A, Petrella F, et al. Volatile signature for the early diagnosis of lung cancer. J Breath Res. 2016;10(1):016007.

147. Westhoff M, Litterst P, Freitag L, Urfer W, Bader S, Baumbach JI. Ion mobility spectrometry for the detection of volatile organic compounds in exhaled breath of patients with lung cancer: results of a pilot study. Thorax. 2009;64(9):744-8.

148. Darwiche K, Baumbach JI, Sommerwerck U, Teschler H, Freitag L. Bronchoscopically obtained volatile biomarkers in lung cancer. Lung. 2011;189(6):445-52.

149. Peled N, Hakim M, Bunn PA, Miller YE, Kennedy TC, Mattei J, et al. Non-invasive breath analysis of pulmonary nodules. J Thorac Oncol. 2012;7(10):1528-33.

150. Poli D, Goldoni M, Caglieri A, Ceresa G, Acampa O, Carbognani P, et al. Breath analysis in non small cell lung cancer patients after surgical tumour resection. Acta Biomed. 2008;79 Suppl 1:64-72.

151. Capuano R, Santonico M, Pennazza G, Ghezzi S, Martinelli E, Roscioni C, et al. The lung cancer breath signature: a comparative analysis of exhaled breath and air sampled from inside the lungs. Sci Rep. 2015;5:16491.

152. Rocco R, Incalzi RA, Pennazza G, Santonico M, Pedone C, Bartoli IR, et al. BIONOTE e-nose technology may reduce false positives in lung cancer screening programmes†. Eur J Cardiothorac Surg. 2015.

153. Dragonieri S, van der Schee MP, Massaro T, Schiavulli N, Brinkman P, Pinca A, et al. An electronic nose distinguishes exhaled breath of patients with Malignant Pleural Mesothelioma from controls. Lung Cancer. 2012;75(3):326-31.

154. Chapman EA, Thomas PS, Stone E, Lewis C, Yates DH. A breath test for malignant mesothelioma using an electronic nose. Eur Respir J. 2012;40(2):448-54.

155. Liu H, Wang H, Li C, Wang L, Pan Z. Investigation of volatile organic metabolites in lung cancer pleural effusions by solid-phase microextraction and gas chromatography/mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;945-946:53-9.

156. Hanai Y, Shimono K, Matsumura K, Vachani A, Albelda S, Yamazaki K, et al. Urinary volatile compounds as biomarkers for lung cancer. Biosci Biotechnol Biochem. 2012;76(4):679-84.

157. Di Lena M, Porcelli F, Altomare DF. Volatile Organic Compounds (VOC) as new biomarkers for colorectal cancer: a review. Colorectal Dis. 2016.

158. Altomare DF, Di Lena M, Porcelli F, Trizio L, Travaglio E, Tutino M, et al. Exhaled volatile organic compounds identify patients with colorectal cancer. Br J Surg. 2013;100(1):144-50.

159. Wang C, Ke C, Wang X, Chi C, Guo L, Luo S, et al. Noninvasive detection of colorectal cancer by analysis of exhaled breath. Anal Bioanal Chem. 2014;406(19):4757-63.

160. Amal H, Leja M, Funka K, Lasina I, Skapars R, Sivins A, et al. Breath testing as potential colorectal cancer screening tool. Int J Cancer. 2016;138(1):229-36.

161. Ma YL, Qin HL, Liu WJ, Peng JY, Huang L, Zhao XP, et al. Ultra-high performance liquid chromatography-mass spectrometry for the metabolomic analysis of urine in colorectal cancer. Dig Dis Sci. 2009;54(12):2655-62.

162. Arasaradnam RP, McFarlane MJ, Ryan-Fisher C, Westenbrink E, Hodges P, Thomas MG, et al. Detection of colorectal cancer (CRC) by urinary volatile organic compound analysis. PLoS One. 2014;9(9):e108750.

163. Westenbrink E, Arasaradnam RP, O'Connell N, Bailey C, Nwokolo C, Bardhan KD, et al. Development and application of a new electronic nose instrument for the detection of colorectal cancer. Biosens Bioelectron. 2015;67:733-8.

164. de Boer NK, de Meij TG, Oort FA, Ben Larbi I, Mulder CJ, van Bodegraven AA, et al. The scent of colorectal cancer: detection by volatile organic compound analysis. Clin Gastroenterol Hepatol. 2014;12(7):1085-9.

165. Batty CA, Cauchi M, Lourenço C, Hunter JO, Turner C. Use of the Analysis of the Volatile Faecal Metabolome in Screening for Colorectal Cancer. PLoS One. 2015;10(6):e0130301.

166. Wang C, Li P, Lian A, Sun B, Wang X, Guo L, et al. Blood volatile compounds as biomarkers for colorectal cancer. Cancer Biol Ther. 2014;15(2):200-6.

167. Xu ZQ, Broza YY, Ionsecu R, Tisch U, Ding L, Liu H, et al. A nanomaterial-based breath test for distinguishing gastric cancer from benign gastric conditions. Br J Cancer. 2013;108(4):941-50.

168. Kumar S, Huang J, Abbassi-Ghadi N, Mackenzie HA, Veselkov KA, Hoare JM, et al. Mass Spectrometric Analysis of Exhaled Breath for the Identification of Volatile Organic Compound Biomarkers in Esophageal and Gastric Adenocarcinoma. Ann Surg. 2015;262(6):981-90.

169. Huang J, Kumar S, Abbassi-Ghadi N, Spaněl P, Smith D, Hanna GB. Selected ion flow tube mass spectrometry analysis of volatile metabolites in urine headspace for the profiling of gastro-esophageal cancer. Anal Chem. 2013;85(6):3409-16.

170. Kumar S, Huang J, Cushnir JR, Španěl P, Smith D, Hanna GB. Selected ion flow tube-MS analysis of headspace vapor from gastric content for the diagnosis of gastro-esophageal cancer. Anal Chem. 2012;84(21):9550-7.

171. Markar SR, Wiggins T, Kumar S, Hanna GB. Exhaled breath analysis for the diagnosis and assessment of endoluminal gastrointestinal diseases. J Clin Gastroenterol. 2015;49(1):1-8.

172. Amal H, Leja M, Funka K, Skapars R, Sivins A, Ancans G, et al. Detection of precancerous gastric lesions and gastric cancer through exhaled breath. Gut. 2015.

173. Buszewski B, Ulanowska A, Ligor T, Jackowski M, Kłodzińska E, Szeliga J. Identification of volatile organic compounds secreted from cancer tissues and bacterial cultures. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;868(1-2):88-94.

174. Zhang Y, Gao G, Liu H, Fu H, Fan J, Wang K, et al. Identification of volatile biomarkers of gastric cancer cells and ultrasensitive electrochemical detection based on sensing interface of Au-Ag alloy coated MWCNTs. Theranostics. 2014;4(2):154-62.

175. Leja M, Amal H, Lasina I, Skapars R, Sivins A, Ancans G, et al. Analysis of the effects of microbiome-related confounding factors on the reproducibility of the volatolomic test. J Breath Res. 2016;10(3):037101.

176. Navaneethan U, Parsi MA, Lourdusamy D, Grove D, Sanaka MR, Hammel JP, et al. Volatile Organic Compounds in Urine for Noninvasive Diagnosis of Malignant Biliary Strictures: A Pilot Study. Dig Dis Sci. 2015;60(7):2150-7.

177. Navaneethan U, Parsi MA, Lourdusamy V, Bhatt A, Gutierrez NG, Grove D, et al. Volatile organic compounds in bile for early diagnosis of cholangiocarcinoma in patients with primary sclerosing cholangitis: a pilot study. Gastrointest Endosc. 2015;81(4):943-9.e1.

178. Qin T, Liu H, Song Q, Song G, Wang HZ, Pan YY, et al. The screening of volatile markers for hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev. 2010;19(9):2247-53.

179. Amal H, Ding L, Liu BB, Tisch U, Xu ZQ, Shi DY, et al. The scent fingerprint of hepatocarcinoma: in-vitro metastasis prediction with volatile organic compounds (VOCs). Int J Nanomedicine. 2012;7:4135-46.

180. Phillips M, Cataneo RN, Ditkoff BA, Fisher P, Greenberg J, Gunawardena R, et al. Volatile markers of breast cancer in the breath. Breast J. 2003;9(3):184-91.

181. Phillips M, Cataneo RN, Ditkoff BA, Fisher P, Greenberg J, Gunawardena R, et al. Prediction of breast cancer using volatile biomarkers in the breath. Breast Cancer Res Treat. 2006;99(1):19-21.

182. Phillips M, Cataneo RN, Saunders C, Hope P, Schmitt P, Wai J. Volatile biomarkers in the breath of women with breast cancer. J Breath Res. 2010;4(2):026003.

183. Phillips M, Beatty JD, Cataneo RN, Huston J, Kaplan PD, Lalisang RI, et al. Rapid point-of-care breath test for biomarkers of breast cancer and abnormal mammograms. PLoS One. 2014;9(3):e90226.

184. Shuster G, Gallimidi Z, Reiss AH, Dovgolevsky E, Billan S, Abdah-Bortnyak R, et al. Classification of breast cancer precursors through exhaled breath. Breast Cancer Res Treat. 2011;126(3):791-6.

185. Patterson SG, Bayer CW, Hendry RJ, Sellers N, Lee KS, Vidakovic B, et al. Breath analysis by mass spectrometry: a new tool for breast cancer detection? Am Surg. 2011;77(6):747-51.

186. Mangler M, Freitag C, Lanowska M, Staeck O, Schneider A, Speiser D. Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting. Ginekol Pol. 2012;83(10):730-6.

187. Barash O, Zhang W, Halpern JM, Hua QL, Pan YY, Kayal H, et al. Differentiation between genetic mutations of breast cancer by breath volatolomics. Oncotarget. 2015.

188. Silva CL, Passos M, Câmara JS. Solid phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers--a powerful strategy for breast cancer diagnosis. Talanta. 2012;89:360-8.

189. Asimakopoulos AD, Del Fabbro D, Miano R, Santonico M, Capuano R, Pennazza G, et al. Prostate cancer diagnosis through electronic nose in the urine headspace setting: a pilot study. Prostate Cancer Prostatic Dis. 2014;17(2):206-11.

190. Roine A, Veskimäe E, Tuokko A, Kumpulainen P, Koskimäki J, Keinänen TA, et al. Detection of prostate cancer by an electronic nose: a proof of principle study. J Urol. 2014;192(1):230-4.

191. Khalid T, Aggio R, White P, De Lacy Costello B, Persad R, Al-Kateb H, et al. Urinary Volatile Organic Compounds for the Detection of Prostate Cancer. PLoS One. 2015;10(11):e0143283.

192. Amal H, Shi DY, Ionescu R, Zhang W, Hua QL, Pan YY, et al. Assessment of ovarian cancer conditions from exhaled breath. Int J Cancer. 2015;136(6):E614-22.

193. Schmutzhard J, Rieder J, Deibl M, Schwentner IM, Schmid S, Lirk P, et al. Pilot study: volatile organic compounds as a diagnostic marker for head and neck tumors. Head Neck. 2008;30(6):743-9.

194. Leunis N, Boumans ML, Kremer B, Din S, Stobberingh E, Kessels AG, et al. Application of an electronic nose in the diagnosis of head and neck cancer. Laryngoscope. 2014;124(6):1377-81.

195. Gruber M, Tisch U, Jeries R, Amal H, Hakim M, Ronen O, et al. Analysis of exhaled breath for diagnosing head and neck squamous cell carcinoma: a feasibility study. Br J Cancer. 2014;111(4):790-8.

196. van Hooren MR, Leunis N, Brandsma DS, Dingemans AC, Kremer B, Kross KW. Differentiating head and neck carcinoma from lung carcinoma with an electronic nose: a proof of concept study. Eur Arch Otorhinolaryngol. 2016;273(11):3897-903.

197. Kwak J, Gallagher M, Ozdener MH, Wysocki CJ, Goldsmith BR, Isamah A, et al. Volatile biomarkers from human melanoma cells. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;931:90-6.

198. Abaffy T, Möller MG, Riemer DD, Milikowski C, DeFazio RA. Comparative analysis of volatile metabolomics signals from melanoma and benign skin: a pilot study. Metabolomics. 2013;9(5):998-1008.

199. Balseiro SC, Correia HR. Is olfactory detection of human cancer by dogs based on major histocompatibility complex-dependent odour components?--A

possible cure and a precocious diagnosis of cancer. Med Hypotheses. 2006;66(2):270-2.

200. Hakim M, Broza YY, Barash O, Peled N, Phillips M, Amann A, et al. Volatile organic compounds of lung cancer and possible biochemical pathways. Chem Rev. 2012;112(11):5949-66.

201. Wang C, Dong R, Wang X, Lian A, Chi C, Ke C, et al. Exhaled volatile organic compounds as lung cancer biomarkers during one-lung ventilation. Sci Rep. 2014;4:7312.

202. Chen X, Xu F, Wang Y, Pan Y, Lu D, Wang P, et al. A study of the volatile organic compounds exhaled by lung cancer cells in vitro for breath diagnosis. Cancer. 2007;110(4):835-44.

203. Filipiak W, Sponring A, Mikoviny T, Ager C, Schubert J, Miekisch W, et al. Release of volatile organic compounds (VOCs) from the lung cancer cell line CALU-1 in vitro. Cancer Cell Int. 2008;8:17.

204. Sponring A, Filipiak W, Mikoviny T, Ager C, Schubert J, Miekisch W, et al. Release of volatile organic compounds from the lung cancer cell line NCI-H2087 in vitro. Anticancer Res. 2009;29(1):419-26.

205. Imhann F, Bonder MJ, Vich Vila A, Fu J, Mujagic Z, Vork L, et al. Proton pump inhibitors affect the gut microbiome. Gut. 2016;65(5):740-8.

206. Shirasu M, Touhara K. The scent of disease: volatile organic compounds of the human body related to disease and disorder. J Biochem. 2011;150(3):257-66.

207. Bikov A, Hernadi M, Korosi BZ, Kunos L, Zsamboki G, Sutto Z, et al. Expiratory flow rate, breath hold and anatomic dead space influence electronic nose ability to detect lung cancer. BMC Pulm Med. 2014;14:202.

208. Queralto N, Berliner AN, Goldsmith B, Martino R, Rhodes P, Lim SH. Detecting cancer by breath volatile organic compound analysis: a review of array-based sensors. J Breath Res. 2014;8(2):027112.

209. Dent AG, Sutedja TG, Zimmerman PV. Exhaled breath analysis for lung cancer. J Thorac Dis. 2013;5 Suppl 5:S540-50.

210. Haick H, Broza YY, Mochalski P, Ruzsanyi V, Amann A. Assessment, origin, and implementation of breath volatile cancer markers. Chem Soc Rev. 2014;43(5):1423-49.

211. Altomare DF, Di Lena M, Porcelli F, Travaglio E, Longobardi F, Tutino M, et al. Effects of Curative Colorectal Cancer Surgery on Exhaled Volatile Organic Compounds and Potential Implications in Clinical Follow-up. Ann Surg. 2015;262(5):862-6; discussion 6-7.

212. Schumer EM, Black MC, Bousamra M, Trivedi JR, Li M, Fu XA, et al. Normalization of Exhaled Carbonyl Compounds After Lung Cancer Resection. Ann Thorac Surg. 2016;102(4):1095-100.

213. Weisburger JH, Reddy BS, Narisawa T, Wynder EL. Germ-free status and colon tumor induction by N-methyl-N'-nitro-N-nitrosoguanidine. Proc Soc Exp Biol Med. 1975;148(4):1119-21.

214. Gagnière J, Raisch J, Veziant J, Barnich N, Bonnet R, Buc E, et al. Gut microbiota imbalance and colorectal cancer. World J Gastroenterol. 2016;22(2):501-18.

215. Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol. 1977;31:107-33.

216. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol. 1999;65(11):4799-807.

217. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiol Rev. 2010;90(3):859-904.

218. Bultman SJ. Interplay between diet, gut microbiota, epigenetic events, and colorectal cancer. Mol Nutr Food Res. 2016.

219. Xu Z, Knight R. Dietary effects on human gut microbiome diversity. Br J Nutr. 2015;113 Suppl:S1-5.

220. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559-63.

221. Bultman SJ. Emerging roles of the microbiome in cancer. Carcinogenesis. 2014;35(2):249-55.

222. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol. 2014;12(10):661-72.

223. Franzosa EA, Morgan XC, Segata N, Waldron L, Reyes J, Earl AM, et al. Relating the metatranscriptome and metagenome of the human gut. Proc Natl Acad Sci U S A. 2014;111(22):E2329-38.

224. Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009;136(1):65-80.

225. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006;7(7):688-93.

226. Xu J, Gordon JI. Honor thy symbionts. Proc Natl Acad Sci U S A. 2003;100(18):10452-9.

227. Stecher B, Hardt WD. The role of microbiota in infectious disease. Trends Microbiol. 2008;16(3):107-14.

228. Boleij A, Tjalsma H. Gut bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. Biol Rev Camb Philos Soc. 2012;87(3):701-30.

229. Sears CL, Garrett WS. Microbes, microbiota, and colon cancer. Cell Host Microbe. 2014;15(3):317-28.

230. Burns MB, Lynch J, Starr TK, Knights D, Blekhman R. Virulence genes are a signature of the microbiome in the colorectal tumor microenvironment. Genome Med. 2015;7(1):55.

231. Flemer B, Lynch DB, Brown JM, Jeffery IB, Ryan FJ, Claesson MJ, et al. Tumour-associated and non-tumour-associated microbiota in colorectal cancer. Gut. 2016.

232. Zackular JP, Rogers MA, Ruffin MT, Schloss PD. The human gut microbiome as a screening tool for colorectal cancer. Cancer Prev Res (Phila). 2014;7(11):1112-21.

233. Zeller G, Tap J, Voigt AY, Sunagawa S, Kultima JR, Costea PI, et al. Potential of fecal microbiota for early-stage detection of colorectal cancer. Mol Syst Biol. 2014;10:766.

234. Yu J, Feng Q, Wong SH, Zhang D, Liang QY, Qin Y, et al. Metagenomic analysis of faecal microbiome as a tool towards targeted non-invasive biomarkers for colorectal cancer. Gut. 2015.

235. Hullar MA, Burnett-Hartman AN, Lampe JW. Gut microbes, diet, and cancer. Cancer Treat Res. 2014;159:377-99.

236. Sears CL, Pardoll DM. Perspective: alpha-bugs, their microbial partners, and the link to colon cancer. J Infect Dis. 2011;203(3):306-11.

237. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. Nat Rev Microbiol. 2012;10(8):575-82.

238. Consortium HMP. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

239. Schwabe RF, Jobin C. The microbiome and cancer. Nat Rev Cancer. 2013;13(11):800-12.

240. Yu YN, Fang JY. Gut Microbiota and Colorectal Cancer. Gastrointest Tumors. 2015;2(1):26-32.

241. Forbes SL, Rust L, Trebilcock K, Perrault KA, McGrath LT. Effect of age and storage conditions on the volatile organic compound profile of blood. Forensic Sci Med Pathol. 2014;10(4):570-82.

242. Berkhout DJ, Benninga MA, van Stein RM, Brinkman P, Niemarkt HJ, de Boer NK, et al. Effects of Sampling Conditions and Environmental Factors on Fecal Volatile Organic Compound Analysis by an Electronic Nose Device. Sensors (Basel). 2016;16(11).

243. Kogan MI, Naboka YL, Ibishev KS, Gudima IA, Naber KG. Human urine is not sterile - shift of paradigm. Urol Int. 2015;94(4):445-52.

244. Kearns B, Whyte S, Chilcott J, Patnick J. Guaiac faecal occult blood test performance at initial and repeat screens in the English Bowel Cancer Screening Programme. Br J Cancer. 2014;111(9):1734-41.

245. Ohigashi S, Sudo K, Kobayashi D, Takahashi O, Takahashi T, Asahara T, et al. Changes of the intestinal microbiota, short chain fatty acids, and fecal pH in patients with colorectal cancer. Dig Dis Sci. 2013;58(6):1717-26.

246. Ohigashi S, Sudo K, Kobayashi D, Takahashi T, Nomoto K, Onodera H. Significant changes in the intestinal environment after surgery in patients with colorectal cancer. J Gastrointest Surg. 2013;17(9):1657-64.

247. Borges-Canha M, Portela-Cidade JP, Dinis-Ribeiro M, Leite-Moreira AF, Pimentel-Nunes P. Role of colonic microbiota in colorectal carcinogenesis: a systematic review. Rev Esp Enferm Dig. 2015;107(11):659-71.

248. Esfahani S, Sagar NM, Kyrou I, Mozdiak E, O'Connell N, Nwokolo C, et al. Variation in Gas and Volatile Compound Emissions from Human Urine as It Ages, Measured by an Electronic Nose. Biosensors (Basel). 2016;6(1).

Appendices

1. Patient information sheet – cancer subjects
2. Patient information sheet – relatives and spouses
3. Consent form – cancer subjects
4. Consent form – relatives and spouses

University Hospitals Coventry and Warwickshire NHS Trust

PARTICIPANT INFORMATION SHEET FOR PARTICIPATION IN RESEARCH PROJECT

Title of the Project: The Use of Electronic Noses (eNOSE) in Assessing Volatile Organic Substances (VOCs) in the Urine and Stools of Colorectal Cancer (CRC) Patients, their Blood Relatives and Individuals with Whom they Share Dwellings

Names of the Investigators: Professor Chuka Nwokolo, Dr R P Arasaradnam, Mr Chris Harmston, Dr James Covington

Invitation

You are being invited to take part in a research study. Before you decide to go ahead, it is important for you to understand why the research is being done and what it will involve. Please take time to read this information carefully and ask us if there is anything that is not clear or if you would like more information.

This study involves

The use of an electronic eNose for non invasive testing and recording of the profile of substances found in your urine and stools. The eNose is pictured below:

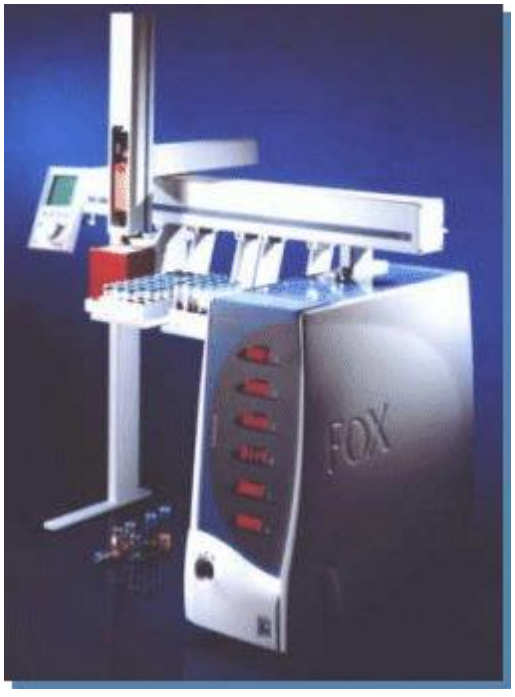


Figure above is an example of an e-nose machine which samples the vapour released by urine and other bodily substances and recognises a unique pattern associated with some diseases.

What happens

You will be recruited on to the study at diagnosis or post CRC resection. You will be asked to provide urine and stool samples to provide us with a record of your profile of substances, using the eNose.

With your permission, we will then contact your first degree relatives (father, mother, brother, sister or child) and/or non blood relatives with whom you share your home, to ask if they would be willing to provide urine and stool samples to provide us with a record of their profile of substances so that we can draw a comparison with yours. They will be contacted by letter and followed up by telephone contact. They will also be offered a face-to-face meeting. A participant information sheet will be sent to them with a consent form to sign and telephone contact will be made for them to ask any questions. Once they return a completed consent form, they will be sent containers for urine (10 ml) and stools (5 ml) along with a secure self addressed envelope for the samples to be returned to us.

What does the study involve?

The aim of the study is to compare the profile of substances found in your urine and stools, to those of your first degree relatives, who share your genes, and/or to those who share your home/environment.

What is the reason for this study?

Preliminary results suggest that patients with cancer produce a unique profile of substances in urine and stools. Presently, approximately 10 to 15% of colorectal cancer is believed to be genetic and over 80% has no clear-cut genetic pattern. There is evidence to suggest that the environment may influence the risk of colorectal cancer i.e. dietary factors. Comparing the patterns in your profile of substances, to those found in your blood relatives and/or non blood relatives you live with, will help to determine the present hypothesis of whether the cause is by “nurture or nature” as well as allowing us to further evaluate the use of the eNose in cancer. These findings could ultimately lead to the first non invasive screening tests for CRC in the UK becoming readily available to the public as a preventative measure.

Do I have to take part?

No, the choice is entirely yours.

What will happen to me if I do take part?

If you do decide to take part, once you have read this information sheet and have had the opportunity to have any questions answered satisfactorily, you will be asked to sign a consent form and we will ask you to provide urine and faecal samples.

You may withdraw at any time without providing an explanation. If you choose not to take part, it will not change any care you may need in the future.

What happens to the samples taken?

We request your permission to store your samples indefinitely as in the future, further techniques may become available to allow us to perform an even more detailed analysis.

Your samples will be kept anonymised so that no-one outside the research team would be able to identify you.

What are the possible benefits of taking part?

There are no immediate benefits for yourself, although future generations of your family could benefit as well as other members of the public. Research of this kind helps to increase the understanding of many diseases, including cancer.

Who has reviewed the study?

West Midlands – Solihull Research Ethics Committee.

Confidentiality

All information we have about you from this study is strictly confidential and will be kept securely for the duration of the study with only the research team having access to it. Any information about you that leaves the hospital as part of a research report will have your name, address and any other personal information removed so that you cannot be identified.

Who is your immediate contact for further information:

Professor Chuka Nwokolo

Honorary Professor of Gastroenterology

Department of Gastroenterology

University Hospitals Coventry & Warwickshire NHS Trust

Clifford Bridge Road

Coventry

CV2 2DX

Direct Tel: 02476966087

Email: chuka.nwokolo@uhcw.nhs.uk or michael.mcfarlane@uhcw.nhs.uk

What happens if something goes wrong?

Nothing is expected to go wrong as all that is required from you is that you post samples by post to us for analysis, therefore nothing can foreseeably go wrong. However, if you do experience any problems you feel are specific to the study that you think we have overlooked, we would appreciate your feedback. In the unlikely event that you wish to make a formal complaint, you can do so by writing to:

Ceri Jones

Head of Research, Development & Innovation

Research, Development & Innovation Department

University Hospitals Coventry & Warwickshire
Clifford Bridge Road

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For independent advice on research, you can contact PALS (Patient Advice and Liaison Service) on freephone 0800 028 4203, Email: PALS@uhcw.nhs.uk

PARTICIPANT INFORMATION SHEET FOR RELATIVES/NON RELATIVES (CONTROL GROUP) WHO LIVE WITH THE PARTICIPANT WHO IS IN THIS RESEARCH PROJECT

Title of the Project: The Use of Electronic Noses (eNOSE) in Assessing Volatile Organic Substances (VOCs) in the Urine and Stools of Colorectal Cancer (CRC) Patients, their Blood Relatives and Individuals with Whom they Share Dwellings (Control Group)

Names of the Investigators: Professor Chuka Nwokolo, Dr R P Arasaradnam, Mr Chris Harmston, Dr James Covington

Invitation

Your relative (our patient) is taking part in a research study and has given us permission to contact you to ask if you would also be willing to take part in the same study. Before you decide to go ahead, it is important for you to understand why the research is being done and what it will involve. Please take time to read this information carefully and ask us if there is anything that is not clear or if you would like more information.

This study involves

The use of an electronic eNose for non invasive testing and recording of the profile of substances found in your urine and stools. The eNose is pictured below:

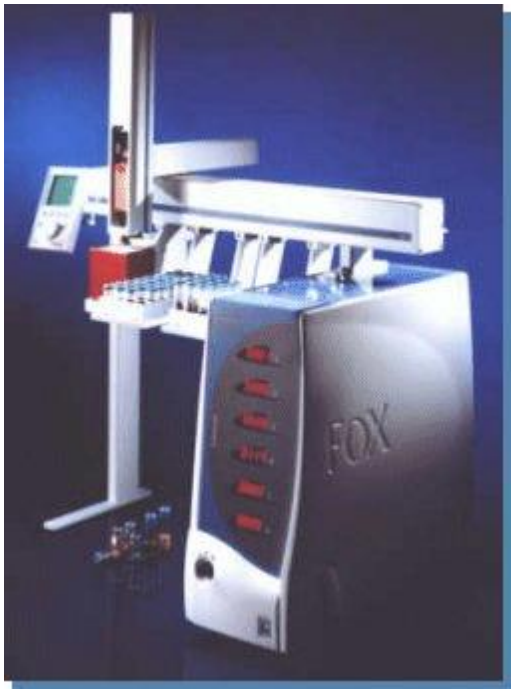

Figure above is an example of an e-nose machine which samples the vapour released by urine and other bodily substances and recognises a unique pattern associated with some diseases.

What happens

You will be asked to provide urine and stool samples to provide us with a record of your profile of substances, using the eNose, so that we can draw a comparison with those of your relative (our patient).

Once you have read this information sheet, we will telephone you so that you can ask any questions, following which, if you wish to take part, we will ask you to sign the consent form sent at the same time as this information sheet. You will also be offered a face-to-face meeting if you prefer. Once you return a completed consent form, you will be sent containers for urine (10 ml) and stools (5 ml) along with a secure self addressed envelope for the samples to be returned to us.

What does the study involve?

The aim of the study is to compare the profile of substances found in your urine and stools, to those of your relative (our patient) as his/her first degree relative, who share his/her genes, and/or as someone who shares his/her home/environment.

What is the reason for this study?

Preliminary results suggest that patients with cancer produce a unique profile of substances in urine and stools. Presently, approximately 10 to 15% of colorectal cancer is believed to be genetic and over 80% has no clear-cut genetic pattern. There is evidence to suggest that the environment may influence the risk of colorectal cancer i.e. dietary factors. Comparing the patterns in your profile of substances, to those found in your relative's (our patient's), will help to determine the present hypothesis of whether the cause is by "nurture or nature" as well as allowing us to further evaluate the use of the eNose in cancer. These findings could ultimately lead to the first non invasive screening tests for CRC in the UK becoming readily available to the public as a preventative measure.

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You may withdraw at any time without providing an explanation. <u>If you choose not to take part, it will not change any care you relative (our patient) may need in the future.</u>

What happens to the samples taken?

We request your permission to store your samples indefinitely as in the future, further techniques may become available to allow us to perform an even more detailed analysis.

Your samples will be kept anonymised so that no-one outside the research team would be able to identify you.

What are the possible benefits of taking part?

There may be no immediate benefits for yourself, although future generations of your family could benefit as well as other members of the public. Research of this kind helps to increase the understanding of many diseases, including cancer.

Who has reviewed the study?

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Confidentiality

All information we have about you from this study is strictly confidential and will be kept securely for the duration of the study with only the research team having access to it. Any information about you that leaves the hospital as part of a research report will have your name, address and any other personal information removed so that you cannot be identified.

Who is your immediate contact for further information:

Professor Chuka Nwokolo

Honorary Professor of Gastroenterology

Department of Gastroenterology

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For independent advice on research, you can contact PALS (Patient Advice and Liaison Service) on freephone 0800 028 4203, Email: PALS@uhcw.nhs.uk

Patient Identification:

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of the project: The Use of Electronic Noses (eNOSE) in Assessing Volatile Organic Substances (VOCs) in the Urine and Stools of Colorectal Cancer (CRC) Patients, their Blood Relatives and Individuals with Whom they Share Dwellings

Names of the Investigators: Professor Chuka Nwokolo, Dr R P Arasaradnam, Mr Chris Harmston, Dr James Covington

	Please Initial (don't tick)	
	Yes	No
1. I confirm that I have read and understand the information sheet dated 12 March 2013 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.		
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.		
3. I understand that giving samples for this research is voluntary and that I am free to withdraw my approval for the use of the samples at any time without giving any reason and without my medical treatment or legal rights being affected.		
4. I agree to give samples of urine and faeces for this research project. I am aware that the samples will be analysed for gaseous products and I understand that the samples given for research purposes will be anonymised and therefore cannot be traced back to myself. Clinical findings will be fed back to my Consultant.		
5. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.		
6. I understand that any personal information obtained as a result of my participation in this study will be treated as confidential and will not be made publicly available.		

7. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.		
8. I agree to take part in the above study.		

Name of the patient	Date	Signature
Name of person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher (original) site file; 1 to be kept in medical notes.

Patient Identification:

CONSENT FORM FOR RELATIVES/NON RELATIVES (CONTROL GROUP) WHO LIVE WITH THE PARTICIPANT WHO IS IN THIS RESEARCH PROJECT

Title of the project: The Use of Electronic Noses (eNOSE) in Assessing Volatile Organic Substances (VOCs) in the Urine and Stools of Colorectal Cancer (CRC) Patients, their Blood Relatives and Individuals with Whom they Share Dwellings

Names of the Investigators: Professor Chuka Nwokolo, Dr R P Arasaradnam, Mr Chris Harmston, Dr James Covington

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	Yes	No
1. I confirm that I have read and understand the information sheet dated 12 March 2013 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.		
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7. I agree to take part in the above study.		

Name of the patient	Date	Signature
Name of person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher (original) site file; 1 to be kept in medical notes.